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EFFECT OF FOOD INGREDIENTS ON THE HUMAN ORAL AND INTESTINAL MICROBIOTA: POLYPHENOLS, PROBIOTICS AND PREBIOTICS

Memoria

que para optar al grado de Doctor Internacional

presenta la Licenciada Elvira Barroso Merinero

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TERESA REQUENA ROLANÍA, DRA. EN VETERINARIA E INVESTIGADORA CIENTÍFICA DEL C.S.I.C. Y MARÍA DEL CARMEN MARTÍNEZ CUESTA, DRA. INGENIERO AGRÓNOMO Y CIENTÍFICA TITULAR DEL C.S.I.C.

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Que el trabajo titulado: **"Effect of food ingredients on the human oral and intestinal microbiota: polyphenols, probiotics and prebiotics**" y del que es autora Elvira Barroso Merinero, ha sido realizado en el Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM), bajo su dirección y cumple las condiciones exigidas para optar al grado de Doctor por la Universidad Autónoma de Madrid y, por tanto, autorizamos su presentación.

Y para que conste, firman el presente Certificado en Madrid a 25 de febrero de 2015

Teresa Requena Rolanía María del Carmen Martínez Cuesta

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La unidad es la variedad, y la variedad en la unidad es la ley suprema del universo.

Isaac Newton

Desgraciados los hombres que tienen todas las ideas claras.

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SUMMARY/RESUMEN

SUMMARY

The currently growing interest in studying the human microbiota highlights its important contribution to health. Microorganisms perform functions critical to host physiology, including metabolism, nutrient absorption and immune and protective functions. Diet plays an important role in the oral microbiota, being at the same time the most significant external factor affecting the intestinal microbiota. Some food ingredients, such as polyphenols, probiotic bacteria and prebiotics have been demonstrated interesting features for approaching microbial modulation studies.

Polyphenols are plant-derived compounds that show an extensive variety of chemical structures, based on which they exert different antimicrobial activities. Likewise, the intestinal microbiota can transform these food compounds into different metabolites. The capability of some bacteria to metabolize polyphenols could provide them with an additional advantage inside the complex intestinal ecosystem and could give rise to more bioactive metabolites with potential beneficial effects on human health. In this respect, *Lactobacillus plantarum* IFPL935 could exert a probiotic effect based on its ability to initiate the metabolism of flaval-3-ols. Other food ingredients, such as prebiotics, selectively promote the composition and/or activity of the intestinal microbiota with beneficial effects on the human body. Currently, there is increasing interest in the search of new prebiotic ingredients able to modulate the microbiota of distal colon regions, usually dominated by proteolytic metabolism.

The objective of this PhD Thesis has been to evaluate the impact of different food ingredients such as polyphenols, potential probiotics such as *L. plantarum* IFPL935 and slow fermentation prebiotics such as lactulose-derived oligosaccharides (OsLu), on the oral and intestinal microbiota.

The comparative study of the impact of different dietary polyphenols on the human intestinal microbiota was performed by batch incubations of colonic microbiota with cranberry, grape seed and red wine polyphenolic extracts. The three phenolic extracts showed antimicrobial activity against the microbiota, being *Bacteroides*, *Prevotella* and *Blautia coccoides-Eubacterium rectale* the most affected groups among those quantified by real time PCR. Grape seed polyphenols showed the highest antimicrobial effect among the three tested extracts.

The modulatory effect of *L. plantarum* IFPL935 on the human intestinal microbiota was studied together with the red wine phenolic extract. There were performed batch incubations during 48 h and a long-term experiment during two weeks, using the dynamic simulator of the human microbiota ecosystem, SHIME. By means of quantitative PCR, we observed in the batch incubations with red wine polyphenols a lower inhibition of *Bacteroides* and *Bifidobacterium* groups in presence of *L. plantarum* IFPL935 compared to the incubations without the strain. The analysis of fermentative metabolism also showed a higher production of butyric acid associated to the presence of *L. plantarum* IFPL935.

The feeding of the SHIME during two weeks with red wine polyphenols exerted an initial antimicrobial effect over the colonic microbiota, being again *Bacteroides* and *B. coccoides/E. rectale* the most affected groups. It could be observed a gradual recovery of counts for all microbial groups through the first days of feeding with the red wine extract. However, the fermentative and proteolytic activities did not recover the initial levels until the second week of feeding with polyphenols. In this context, *L. plantarum* IFPL935 showed a protective role against the antimicrobial effect on the butyrate-producing groups, *Ruminococcus*, *B. coccoides/E. rectale* and *Clostridium leptum*. Likewise, *L. plantarum* IFPL935 had a positive impact on the butyric acid production in distal colon regions.

Concerning polyphenols metabolism, in presence of the complex human colonic microbiota, *L. plantarum* IFPL935 was able to initiate the metabolism of flavan-3-ols, detected by the significant increase of intermediate compounds such as diphenylpropan-2-ol, (5-(3′-hydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3’-hydroxyphenyl)-valeric acid, and favoured the formation of end metabolites such as phenylpropionic acids.

With the aim of evaluating the effect of moderate red wine intake on the composition of the oral and intestinal microbiota, it was performed a human intervention study during four weeks. Both, quantitative PCR and PCR-DGGE analysis of the salivary microbiota and metagenomic analysis of the faecal samples from the volunteers, did not show significant changes on the composition of the microbiota from both environments. The most predominant genera detected in saliva were *Streptococcus* (8 log bacteria/mL) and *Lactobacillus* and *Prevotella* (7 log bacteria/mL). In faeces, they were detected 2324 phylotypes, of which 30 were found over the 0.5% of mean relative frequency, representing an 84.6% of the total taxonomical assignments and bellowing to Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria phyla. Shannon index calculation showed an increase in microbial diversity of faecal samples after wine intake. However, the large inter-individual variability observed in the profiles of the intestinal microbiota exerted a stronger influence for the grouping of samples by individuals than the possible influence of wine intake.

In order to unravel the effect of slow fermentation prebiotics on the human microbiota differentiated by colonic regions, it has been developed a new dynamic automatic *in vitro* gastrointestinal simulator (SIMGI). The computer-controlled system allows simulating the physiologic process of digestion and fermentation inside the human gastrointestinal tract. In addition, in this work, it has been demonstrated the capability of the

SIMGI to stable reproduce the composition and fermentative activity of the ascending, transverse and descending colon regions of the human microbiota. Thus, this system allowed performing the evaluation of the prebiotic effect of OsLu on the region-specific colon human microbiota. The stabilization of the human faecal microbiota inoculated in the SIMGI and differentiated by colonic regions was reached by feeding the system with a high energy diet based on the high content of fructose and easily fermentable carbohydrates. The quantitative PCR results did not show significant shifts in the composition of the microbiota due to the feeding with OsLu, but it allowed maintaining the microbial fermentative functionality, represented by net butyric acid production in the ascending colon region. In addition, it was avoided a full transition to proteolytic metabolism profiles, which took place when changing to a diet in which easily fermentable carbohydrates were removed.

RESUMEN

El creciente interés dedicado al estudio de la microbiota humana ha puesto de manifiesto su importante contribución en la salud. Los microorganismos desempeñan funciones críticas para la fisiología del hospedador, incluyendo el metabolismo, la absorción de nutrientes y funciones inmunológicas y protectoras. La dieta juega un papel significativo en la microbiota oral, siendo a la vez el factor externo más relevante que afecta a la microbiota intestinal. Algunos ingredientes alimentarios, como los polifenoles, las bacterias probióticas y los prebióticos, poseen características que los hacen interesantes para abordar estudios de modulación de la microbiota oral e intestinal.

Los polifenoles son compuestos presentes en las plantas que poseen una amplia variedad de estructuras químicas y en función de las mismas muestran cierta variabilidad en su actividad antimicrobiana. Así mismo, la microbiota intestinal puede transformar estos compuestos de los alimentos en diferentes metabolitos. La capacidad de algunas bacterias para metabolizar polifenoles puede aportarles ventajas dentro del ecosistema intestinal, además de favorecer la formación de metabolitos bioactivos beneficiosos para el organismo. En este sentido, *Lactobacillus plantarum* IFPL935 podría ejercer un efecto probiótico ya que posee la capacidad de iniciar el metabolismo de flavan-3-oles. Otros ingredientes alimentarios, como los prebióticos, favorecen selectivamente la composición y/o actividad de la microbiota intestinal que ejerce beneficios en la salud. En la actualidad se tiende a la búsqueda de nuevos prebióticos que puedan modular la microbiota de zonas distales del colon que suelen estar dominadas por un metabolismo proteolítico.

El objetivo de la Tesis Doctoral ha sido evaluar el impacto de diferentes ingredientes alimentarios como polifenoles, potenciales probióticos como *L. plantarum* IFPL935 y prebióticos de fermentación

lenta como oligosacáridos de lactulosa (OsLu) sobre la microbiota oral e intestinal humana.

La comparación del impacto de diferentes polifenoles de la dieta en la microbiota intestinal se realizó mediante incubaciones en condiciones estáticas de microbiota colónica con extractos de polifenoles de arándano rojo, pepita de uva y vino tinto. Los tres extractos fenólicos mostraron actividad antimicrobiana frente a la microbiota, siendo *Bacteroides*, *Prevotella* y *Blautia coccoides-Eubacterium rectale* los grupos bacterianos más afectados de los cuantificados por PCR a tiempo real. El extracto de pepita de uva mostró la mayor actividad antimicrobiana de los tres extractos estudiados.

El efecto modulador de la cepa *L. plantarum* IFPL935 sobre la microbiota intestinal se estudió junto con el extracto de polifenoles de vino tinto. Se realizaron incubaciones tanto en condiciones estáticas durante 48 h, como durante dos semanas en el simulador dinámico del ecosistema microbiano intestinal humano SHIME. Mediante cuantificación por PCR a tiempo real, en las incubaciones estáticas con polifenoles de vino tinto se pudo observar una menor inhibición de los grupos bacterianos *Bacteroides* y *Bifidobacterium* en presencia de *L. plantarum* IFPL935. El análisis del metabolismo fermentativo también mostró una mayor producción de ácido butírico asociada a la presencia de *L. plantarum* IFPL935.

La alimentación del SHIME durante dos semanas con el extracto de polifenoles de vino tinto ejerció un efecto antimicrobiano inicial sobre la microbiota colónica, observándose de nuevo la mayor inhibición frente a *Bacteroides* y *B. coccoides/E. rectale*. Se pudo observar una recuperación progresiva de los recuentos de todos los grupos microbianos durante los primeros días de administración del extracto de vino tino. Sin embargo, las actividades fermentativa y proteolítica no recuperaron los niveles iniciales hasta la segunda semana de suministro

de polifenoles. En este contexto, *L. plantarum* IFPL935 mostró un papel protector del efecto antimicrobiano sobre los grupos productores de ácido butírico *Ruminococcus*, *B. coccoides/E. rectale* y *Clostridium leptum*. Asimismo, *L. plantarum* IFPL935 ejerció un impacto positivo sobre la formación de ácido butírico en regiones colónicas distales.

En relación al metabolismo de polifenoles, *L. plantarum* IFPL935 en presencia de microbiota intestinal inició el metabolismo de flavan-3-oles, detectado por el aumento significativo de la concentración de compuestos intermediarios como dihidroxifenilpropan-2-ol, 5-(3′-hidroxifenil)-γ-valerolactona y ácido 4-hidroxi-5-(3′-hidroxyfenil)valérico, y favoreció la formación de metabolitos finales como ácidos fenilpropiónicos.

Con objeto de evaluar el efecto del consumo moderado de vino tinto sobre la microbiota oral e intestinal, se llevó a cabo un estudio de intervención en humanos durante un periodo de cuatro semanas. Tanto el análisis por PCR cuantitativa y PCR-DGGE de la microbiota de la saliva de los voluntarios como el análisis metagenómico de sus muestras fecales, mostraron que no hubo cambios significativos en la composición de las poblaciones bacterianas. Los generos mayoritarios detectados en saliva fueron *Streptococcus* (8 log bacterias/mL) y *Lactobacillus* y *Prevotella* (7 log bacterias/mL). En heces, se detectaron más de 2324 filotipos, de los cuales 30 tenían frecuencias relativas medias por encima del 0,5%, representaban el 84,6% del total de las asignaciones taxonómicas y pertenecían a los filos Firmicutes, Actinobacteria, Bacteroidetes y Proteobacteria. El cálculo del índice de Shannon mostró un aumento de la biodiversidad en las muestras de heces de los voluntarios tras el consumo de vino. Sin embargo, la alta variabilidad interindividual observada en los perfiles de la microbiota intestinal tuvo mayor efecto en el agrupamiento por individuos que el que pudiera ejercer el consumo de vino.

Para evaluar el efecto de ingredientes alimentarios, particularmente de prebióticos de fermentación lenta, sobre la microbiota humana diferenciada en regiones colónicas, se ha llevado a cabo el desarrollo de un nuevo modelo dinámico automatizado de simulación gastrointestinal *in vitro* (SIMGI). El sistema está controlado por ordenador y permite simular los procesos fisiológicos de digestión y fermentación que tienen lugar en el tracto gastrointestinal humano. Además, en este estudio se ha demostrado la capacidad del SIMGI para reproducir de manera estable la composición y la actividad fermentativa de la microbiota representativa de las regiones del colon ascendente, transverso y descendente. El simulador permitió, por tanto, llevar a cabo la evaluación del efecto prebiótico de los OsLu en la microbiota humana de distintas regiones del colon. La estabilización de la microbiota fecal humana inoculada en el SIMGI y diferenciada por regiones colónicas se alcanzó alimentando el sistema con una dieta con alto contenido energético, rica en fructosa y carbohidratos fácilmente fermentables. Los resultados de cuantificación microbiana por PCR a tiempo real no mostraron variaciones significativas en la composición de la microbiota debidas a la alimentación con OsLu, pero permitió mantener la funcionalidad fermentativa de la microbiota, reflejada en la producción neta de ácido butírico en la región ascendente del colon. Además, se evitó la transición completa a perfiles metabólicos proteolíticos que tuvieron lugar en el cambio a una alimentación en la que se eliminaron los carbohidratos fácilmente fermentables.

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I. INTRODUCTION

I.1 THE HUMAN MICROBIOTA

Humans harbour densely resident microbial communities including bacteria, fungi, parasites and viruses refereed as microbiota and the genes they encode are known as microbiome (Hooper et al., 2010; Clemente et al., 2012). These human microbial populations contain more than 100 times the number of genes and 10 times the number of cells compared with the host (Qin et al., 2010; Huttenhower et al., 2012).

Different sites in humans are colonized by microbial communities during neonatal and childhood development and throughout the lifetime of individuals. Each human habitat harbours a characteristic microbiota represented by a set of abundant taxa that is stable between individuals and over time (Reid et al., 2011). (Figure I.1). The oral cavity is densely populated with 10^8-10^{11} bacteria/g in dental plaque, somewhat higher than vagina with 10^8-10^9 bacteria/mL (Costello et al., 2009; Lamont et al., 2011; Zhou et al., 2013). The skin is generally low densely populated, but regions such as the axillae and the perineum support much more microbes in number but less diversity (Grice and Segre, 2011; Callewaert et al., 2013). The upper regions of the respiratory tract also harbour high density of bacteria which markedly decrease to reach the bronchi and alveoli (Charlson et al., 2011). Within the gastrointestinal tract, the population densities and communities also vary depending on the site: the stomach, duodenum, and ileum have low population densities, whereas the jejunum, caecum, and colon are densely populated, 10^{11}-10^{12} bacteria/g (Huttenhower et al., 2012). Overall, the oral cavity and colon show by far the highest density of bacterial populations in the whole body. (Figure I.1)

Many of these microorganisms such as those inhabiting the skin, oro-gastrointestinal or vaginal tract have co-evolved with humans along millions of years and show a mutualistic relationship with them. In most cases, healthy humans get used to their microbiota and either friendly

tolerates it and/or get profit on it. Microorganisms perform functions critical to host physiology, including metabolism, nutrient absorption and immune and protective functions (Walsh et al., 2014), while in turn, the host supplies niches and nutrients to the microbiota.

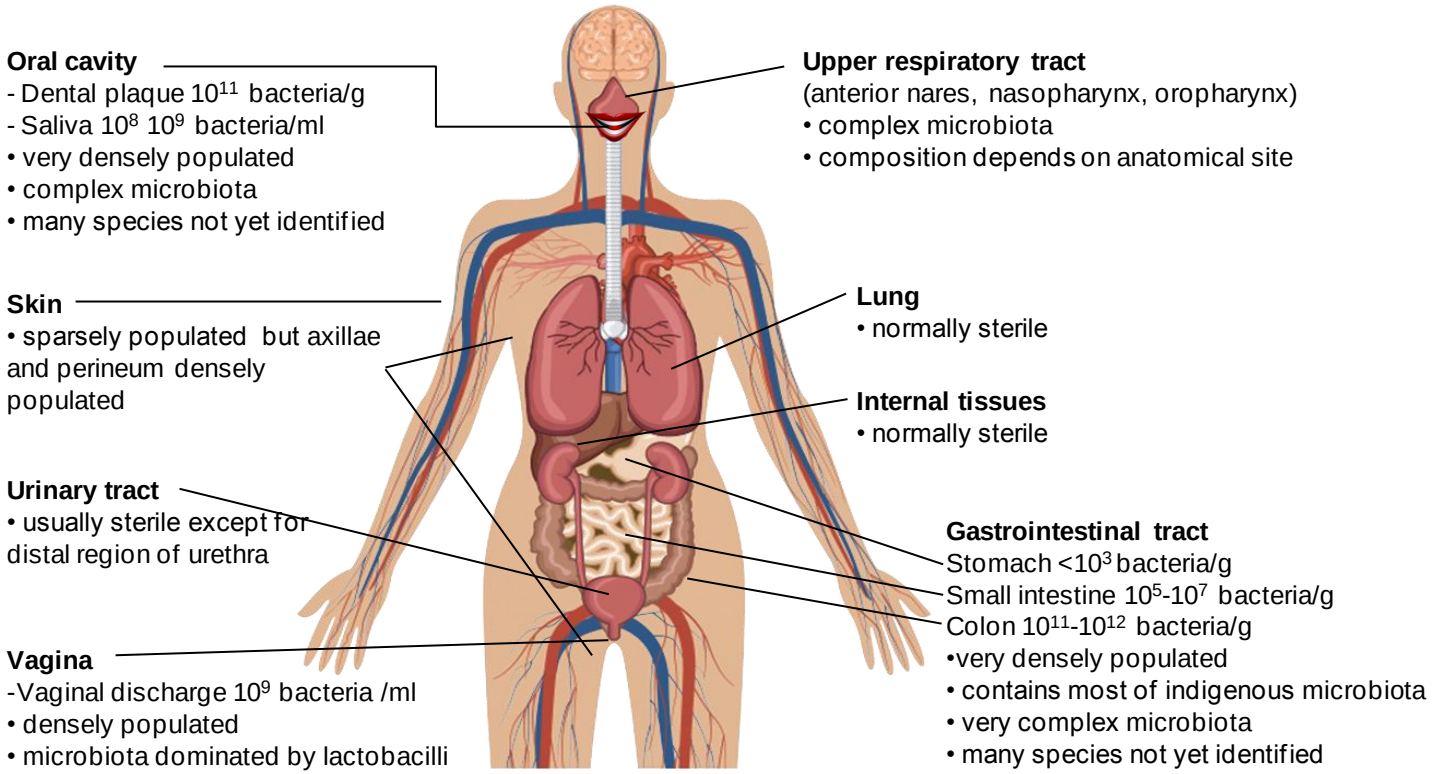


Figure I.1. Distribution of the indigenous microbiota of the human body.

I.1.1 Acquisition and development of oral and intestinal microbiota

In classic studies about acquisition of the intestinal microbiota (Tissier, 1900), it was stated that the body is sterile before birth. Nevertheless, there is evidence of bacteria presence in placenta, umbilical cord, amniotic fluid and meconium in healthy neonates (Jiménez et al., 2008; Moles et al., 2013; Stout et al., 2013; Aagaard et al., 2014). It has been hypothesized a process for transporting bacteria from the mother oral cavity to the placenta during pregnancy carried out by the blood (Zaura et al., 2014), based on the fact that human oral microbiota was found to be most akin to the placenta microbial profiles than the vaginal and faecal microbiota (Aagaard et al., 2014). These early microbial exposure of the foetus to maternal oral microbiota in placenta could represent a mechanism of "training" for the foetal immune system (Aagaard et al., 2014; Zaura et al., 2014).

During and following birth, neonates become colonized by a vast array of microorganisms from the mother´s birth canal or skin, or from other environmental sources (Zaura et al., 2014). Vertical transmission from the mother to child during birth affects the diversity of the oral microbiota. Vaginally delivery mode has showed to positively affect the microbial diversity of children at 3 months of age (Holgerson et al., 2011). In addition, Li and colleagues have reported that oral microbiota of infants born with caesarean section acquired *Streptococcus mutans* almost one year earlier than vaginally born infants (Li et al., 2005); this species has been identified as a significant contributor to tooth decay. On the other hand, in the oral cavity the first persistent colonizers dominating nearly all oral mucosal sites are other species of *Streptococcus* (*S. oralis*, *S. mitis*, *S. peroris* and *S. salivarius*) (Zaura et al., 2014). Breast feeding also affects infant microbiota. It has been shown that *Lactobacillus* colonized the oral cavity of breastfed infants significantly more frequently than

formula fed infants, being *L. gasseri* the most prevalent species, which has showed probiotic properties that could influence the composition of the oral microbiota in infants (Vestman et al., 2013). The range of microorganisms that can be isolated from the oral cavity increases following the emergence of teeth and along the life. Horizontal transmission of microbiota among people from the same environment also affects oral microbiota diversity (Zaura et al., 2014). Local alterations in the environment, due to microbial activity (oxygen consumption, pH changes, etc.) and the provision of new sites for colonization and nutrients (bacterial cells themselves), promote the establishment of the adult-like microbial community. The oral cavity has two main types of surfaces for microbial colonization: non-shedding (teeth) and shedding (mucus). Studies of the oral cavity have shown that these different structures are colonized by distinct microbial communities (Marsh and Percival, 2006). Once established, the health microbiome should be sustained. In adults, factors such as correct hygiene permit a correct balance between host and microorganisms that helps to maintain oral health (Jenkinson and Lamount, 2005; Marsh and Percival, 2006). Under these conditions, dental plaque (in which microorganisms form a dynamic and complex ecosystem called biofilm) can have a protective role; however, it could also have a role as a reservoir of bacteria involved in caries and periodontitis (Jenkinson and Lamount, 2005; Marsh and Percival, 2006). The term supragingival plaque refers to those biofilms that are formed on the tooth surface above the level of the gingival margin. It could contain as much as 10^{11} bacteria/g of plaque, especially in patients with periodontitis (Marsh and Devine, 2011). In comparison to teeth and tongue, saliva presents a lower microbial load, containing approximately 10^{8} bacteria /mL.

The same as oral microbiota, the gastrointestinal microbiota is structured mainly by the mode of birth delivery. Infants born vaginally have communities resembling their own mother’s vaginal microbiota,

dominated by *Lactobacillus*, *Prevotella* or *Sneathia* (Reid et al., 2009; Reid et al. 2011). In contrast, those delivered by caesarean section harbour a microbiota characteristic of the skin communities of the mother, and are dominated by *Staphylococcus*, *Corynebacterium* and *Propionibacterium* (Reid et al., 2011). Since the intestine of the newborn contains oxygen, this initial colonization is slowly progressing towards dominance of facultative anaerobes, such as members of the *Enterobacteriaceae* family. Later, when gut turns out completely anaerobic, strict anaerobic bacteria dominate the microbial community, such as *Bifidobacterium*, *Clostridium* and *Bacteroides*. Furthermore, the composition of the intestinal microbiota can greatly differ between infants that are fed with either breast milk or formula. Human-breast milk contains its own microbial consortium (Hunt et al., 2011), along with non-digestible oligosaccharides (human milk oligosaccharides, HMOs), maternal antibodies (IgA) and antimicrobial factors. In this regard, *Bifidobacterium* dominate the gut of breast-fed infants while formula fed infants show higher proportions of *Bacteroides* and *Clostridium* (Fallani et al., 2010; Bezirtzoglou et al., 2011). Furthermore, due to the bacterial DNA similarities found between human milk, maternal faecal samples, infant faeces and maternal peripheral blood mononuclear cells, it has been suggested that intestinal bacteria could migrate via the blood and lymph circulation to the mammary gland, for the formation of the breast milk microbiome. This represents a similar transport mechanism of that of the maternal oral microbiota to placenta (Jeurinketal et al., 2013).

The introduction of solid food represents another important shift in the gut microbiota. It has been reported two distinct phases in gut colonization after birth separated by the introduction of solid foods (Vallés et al., 2014). Nutrients that become available to the infant gut microbiota strongly increase with solid foods, including numerous complex carbohydrates found in cereals, fruits, vegetables and tubers, providing new substrates that contribute to the increase in diversity. This is shown

in the rise of *Bacteroides* observed in 7-months infants (Vallés et al, 2014). Furthermore, some new genera start to be detected, this is the case of *Ruminococcus*, which thrives on oligosaccharides (Cervera-Tison et al., 2012) and *Akkermansia*, a mucin degrader genus linked to the presence of new sources of protein (Derrien et al., 2010). *Bifidobacterium*, *Veillonella* and *Escherichia* decrease at one year old age, and short chain fatty acids (SCFAs) producers, such as *Blautia*, *Butyrivibrio*, *Faecalibacterium*, *Eubacterium*, *Roseburia* and *Clostridium leptum*, rise in abundance at this time. It has been suggested that the main core of the gut community is established at the age of one year; however, recent cross sectional studies have suggested that an adult-like gut community is not reached before three years of age (Yatsunenko et al., 2012). Until this age the microbiota of children shows larger inter-individual variability and lower diversity than in adults (Lozupone et al., 2012). It has been observed that changes in the microbiota in early life, due among other factors to antibiotic use, affect the microbial composition and susceptibility to immunological diseases into adulthood, although the biological mechanisms are not well understood (Kozyrskyj et al., 2011).

Despite the highly unstable early stage of microbial colonization, the establishment of the adult intestinal ecosystem seems to follow a conventional program in healthy conditions (El Aidy et al., 2013). Due to the physiological and microbial changes in the intestinal environment, it leads to the final colonization of a robust and stable microbiota dominated by Firmicutes, Bacteroidetes and Actinobacteria, able to develop the mutualistic relation with the host, needed to maintain the intestinal homeostasis (Bäckhed et al., 2005; Yatsunenko et al., 2012). However, during pregnancy it has been shown an increase in Actinobacteria and Proteobacteria, but the gut microbiota returns to its original structure after delivery (Koren et al., 2012). Furthermore, elderly (>65 years old) are associated with an increase in the abundance of Bacteroidetes and a decrease in diversity and displays greater inter-individual variability than

that of younger adults (Claesson et al., 2011). The inter-individual variation in microbiota composition of elderly is associated to residence location, long-term stay in hospitals or rehabilitation clinics and antibiotics usage, among others (Claesson et al., 2011).

I.1.2 Community composition and diversity of oral and intestinal microbiota

I.1.2.1 Culture-independent techniques for studying the human microbiota

Our understanding of the indigenous human microbiota and its composition, how it interacts with the host and how it maintains the balance in human health or causes disease, has been enhanced by advances in culture-independent techniques (Fraher et al., 2012). For many years, culture and biochemical typing have been very useful and even essentials to identify bacterial species. However, since the 1990s the development of culture-independent techniques based on 16S rRNA gene sequences has revolutionized our knowledge and helped to evaluate the composition of the human microbiota (Rajilić-Stojanović et al., 2007; Ventura et al., 2009; Marchesi, 2011). These culture-independent techniques have allowed demonstrating the microbial diversity of the human microbiota, providing qualitative and quantitative knowledge on bacterial species and changes in the microbiota in relation to health and disease (Fraher et al., 2012). Examples of these techniques are denaturing gradient gel electrophoresis (DGGE), quantitative polymerase chain reaction (qPCR), DNA microarrays, sequencing of cloned 16S rRNA gene amplicons and next-generation sequencing of the 16S rRNA genes and microbial genomes (Table I.1). These next-generation methods are typically applied to DNA amplicons directly or to the total genomic DNA. They are described as massively parallel sequencing as ‘massive’ numbers of DNA templates can be sequenced in

parallel; that is, at the same time and in the same reaction set-up, a very large amount of short sequences can be read and thus even bacteria that are in low abundance can be detected (Rogers and Venter 2005, Fraher et al., 2012). Commercially available technologies include 454 Pyrosequencing®, Illumina®, SOLiD™ and Ion Torrent™ (Table I.1). The term metagenomics in microbiology refers to the study of the collective genomes of the microbiota from a site (named microbiome). Thus the metagenomic studies consist of sequencing all the DNA of a sample rather than a particular DNA fragment (Sekirov et al., 2010). Large metagenomic studies are that of The Human Microbiome Project, which has studied the structure, function and diversity of the healthy human microbiome from hundreds of samples and their correlations with diet and age (Huttenhower et al., 2012) and the Metagenomics of the Human Intestinal Tract (MetaHIT) project that has studied the metagenomic profile of hundreds of faecal samples from healthy adults, overweight/obese individuals and intestinal bowel disease patients (Qin et al., 2010; Arumugam et al., 2011). Due to the increasingly easy access of these culture-independent technologies, there is currently an increase of research groups performing broad spectrum studies of the human microbiota for different purposes, most of them related with health (Thomas et al., 2014).

Metagenomic approaches have started to put forward the microbial functionalities embedded into the human microbiota (Lepage et al., 2013). The easy access to the oral cavity has allowed sampling and therefore obtaining much information of the microbiota from the different surfaces inside on it, such as buccal mucosa, subgingival and supragingival plaque, saliva, tongue, etc. (Wade, 2013; Zaura et al., 2014). However, most of the studies about composition, diversity and functions of the human gut microbiota have been conducted with faecal material (De Filippo et al., 2010; Wu et al., 2011; Huttenhower et al., 2012; Methé et al., 2012; Voreades et al., 2014), due to the easy and non-invasive

methods required to obtain these samples. It is generally accepted that faecal samples could represent luminal microbiota; however, mucosal associated microbiota, as well as the microbial communities present in the different colonic regions, are not represented, being only accessible by invasive methods so far (Watt et al., 2013).

Table I.1. Techniques for studying the human microbiota.

Technique	Description	Advantages	Disadvantages
Culture	Selective media for isolation of bacteria.	Semi-quantitative, cheap.	Limited to certain bacterial groups, time consuming.
Quantitative - PCR	Amplification and quantification of 16S rRNA. The PCR product is labeled with a fluorochrome reporter. Fluorescence is directly proportional to the amount of product.	High sensitivity, quantitative, fast, phylogenetic identification and gene expression.	No identification of unknown species, PCR bias.
DGGE/TGGE (denaturing gradient gel electrophoresis/ temperature gradient gel electrophoresis)	Gel separation of 16S rRNA amplicons in bands using denaturant/temperature. Obtaining a molecular fingerprint. Each band represents one specie.	Semi-quantitative, bands can be excised and sequenced.	No phylogenetic identification, PCR bias.
FISH (fluorescence in situ hybridization)	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry.	Phylogenetic identification, semi-quantitative, no PCR bias, can easily differentiate between live and dead cells.	Dependent on probe sequences, unable to identify unknown species.
DNA microarrays	Fluorescently labelled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser.	Phylogenetic identification, semi-quantitative, fast, simultaneous identification of thousands of genes.	Cross hybridization, PCR bias, species present in low levels can be difficult to detect.
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, sanger sequencing and capillary electrophoresis.	Phylogenetic identification, quantitative.	PCR bias, laborious, expensive, cloning bias.
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons for example, 454 Pyrosequencing® (Roche Diagnostics GMBH Ltd, Mannheim, Germany) (amplicon immobilized on beads, amplified by emulsion PCR, addition of luciferase results in a chemoluminescent signal)	Phylogenetic identification and biodiversity analysis, quantitative, fast, identification of unknown bacteria, not need to insert gene fragments in a host.	PCR bias, expensive, laborious.
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome (e.g. 454 pyrosequencing®, Illumina®, Ion Torrent™)	Phylogenetic identification, quantitative, whole-genome sequencing, allows analysis of biodiversity and functionality.	Expensive, analysis of data is computationally intense.

Adapted from Fraher et al., 2012.

I.1.2.2 Oral microbiota

More than 200 microbial species from the oral cavity have been isolated using classical culture techniques, while culture-independent molecular methods primarily using 16S rRNA gene-based cloning studies have identified approximately 600 more phylotypes (Dewhirst et al., 2010). Today, it is thought that the number may reach 10000 phylotypes as revealed by pyrosequencing analyses (Keijser et al., 2008; Zaura et al., 2009; Diaz, 2011). The bacterial community of the mouth is dominated by the phyla Firmicutes (*Streptococcus*, *Veillonella*ceae, *Granulicatella*), Proteobacteria (*Neisseria*, *Haemophilus*), Actinobacteria (*Corynebacterium*, *Rothia*, *Actinomyces*), Bacteroidetes (*Prevotella*, *Capnocytophaga*, *Porphyromonas*), Spirochaetes and Fusobacteria (*Fusobacterium*) (Lazarevic et al., 2009; Zaura et al., 2009; Lazarevic et al., 2010), which account for 96% of the species detected (Dewhirst et al., 2010). The proportion of the microbiota that has been cultivated (approximately 40%) is higher for the oral cavity than for many other body sites. This reflects both the ease of access to samples for analysis and the great interest shown in the oral microbiota over time due to its role in two of the most common infections of humans, caries and periodontal diseases (Fitzgerald and Keyes, 1960; Bradshaw et al,. 2013). The main species on supragingival plaque belong to *Streptococcus* (mainly *S. sanguis*, *S. oralis* and *S. mitis*), *Neisseria*, *Haemophilus* and *Actinomyces* (Marsh and Devine, 2011). Recent studies of salivary microbiota in adults have revealed a characteristic microbial community with certain stability and a persistence of subject-specificity (Lazarevic et al., 2010; Stahringer et al., 2012; Romano-Keeler et al., 2014). Despite the variety of habitats and microbial communities in the oral cavity, there are species common to all sites that have been reported to belong to the genera *Streptococcus*, *Abiotrophia*, *Gemella*, *Granulicatella* and *Veillonella* (Dewhirst et al., 2010).

I.1.2.3 Intestinal microbiota

The intestinal microbiota is a complex and dynamic community that contains a diverse range of microorganisms. The different regions of the intestinal tract vary widely in terms of transit time, pH, oxygen and nutrient availability, host secretions, mucosal surfaces and interactions with the immune system, all of which affect microbial composition. Metagenomic studies have shown that the majority of gut microbiota sequences belong to bacteria (Eckburg et al., 2005; Qin et al., 2010; Arumugam et al., 2011). Currently, only a small minority of these bacteria -over 400 species- has been successfully isolated and cultured (Eckburg et al., 2005; Rajilić-Stojanović et al., 2007). More recently, culture-independent techniques have indicated that well over 1000 species are capable of colonizing the human gut (Huttenhower et al., 2012). Despite the high density of bacteria in the human gut and their complexity, diversity at the division level is low. Firmicutes, Bacteroidetes and Actinobacteria represent the dominant phyla, being 60-90% of the total population (Neish, 2009; Walker at el., 2011). The rest of the dominating bacterial phyla correspond to Proteobacteria, Verrucomicrobia and Fusobacteria (Eckburg et al., 2005; Tap et al., 2009; Arumugam et al., 2011). Within the Firmicutes phylum, 95% of the phylogenetic types are members of the Clostridia class, and much of them are related to butyrate-producing bacteria, all of which fall within the clostridial clusters IV, XIVa, and XVI (Eckburg et al., 2005; Tap et al., 2009). Other bacteria commonly found in the human gut at genus level are *Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, *Roseburia*, *Alistipes* and *Lactobacillus* that vary depending on age and individuals (Arumugam et al., 2011; Mueller et al., 2006; Frank et al., 2007). Although there is a huge inter-individual variability in the intestinal microbiota composition, it has been suggested that the microbiota of most individuals can be categorized into one of three variants or ‘‘enterotypes’’ clusters, that are not nation or continent specific, dominated by *Bacteroides*, *Prevotella* and *Ruminococcus*,

respectively (Arumugam et al., 2011). These clusters may in fact be more appropriately characterized as a ratio of the abundance of *Bacteroides* and *Prevotella*, with the *Ruminococcus* enterotype folded into the *Bacteroides* group (Wu et al., 2011). These broad patterns were strongly associated with long-term diets, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*) (Wu et al., 2011); it remains to be seen how important they are in understanding overall microbial community functions. More recent studies, however, suggested rethinking about the term enterotypes, as most human gut microbiome data collected to date support continuous gradients of dominant taxa rather than discrete enterotypes; furthermore, an individual's enterotype can be highly variable (Jeffery et al., 2012; Knights et al., 2014).

I.1.3 Functions of the oral and intestinal microbiota: the impact on human health

It is clear that the mutualistic relationship between the microbiota and the human host is essential in health maintenance (Wade, 2013; Zaura et al., 2014), but these interactions may vary according to the individual. Moreover, the genetic background, age, diet and health status of the host affect the activity and composition of the microbiota (Ottman et al., 2012).

I.1.3.1 Oral microbiota

The first function of the oral cavity during food ingestion is informing our brain of the palatability and acceptance of the food, as well as its processing by mechanical and hydrodynamic forces into suitable forms further digested in the gastrointestinal tract. In addition, the concerted action of different enzymes (amylase, ß-glucosidases, peptidases and esterases) from salivary glands, epithelial cells and/or bacteria, could initiate the digestion process of multiple diet components and permit growth of bacteria using saliva and mucus as a nutrient source. Further,

within the complexity of oral biofilms, the microbial species form communities with metabolic functions and inter- and intra-species interactions, and gain significant advantages such as protection against host defences and antimicrobial agents (Marsh and Devine, 2011; Wade, 2012). Moreover, there is a host–microbiota cross talk during biofilm development that allows establishment of a healthy balance (De Ryck et al., 2014).

The function of the oral microbiota leads to the interactions between beneficial or potentially pathogen oral bacteria, which have been divided into competition for nutrients, synergy, antagonism, neutralization of virulence factors and interference in signaling mechanisms (Kuramitsu et al., 2007). The maintenance of the functional stability and homeostasis along the life also depends on bidirectional interactions between the microbiota and the host (Zaura et al., 2014). Besides, it has been stated that the complex oral ecosystem, and not a unique bacteria, is responsible for the development of dental caries and other oral diseases (Jenkinson and Lamont, 2005; Siqueira and Rôças, 2009).

Oral surfaces are continuously bathed in saliva, which contains a characteristic bacterial community, represents a nutrient source and keeps the pH close to neutrality. Thus, the saliva also plays an important role in the development and maintenance of the healthy microbial ecosystem (Stahringer et al., 2012; Romano-Keeler et al., 2014) and it is considered useful as a disease indicator (Mager et al., 2005; Lazarevic et al., 2010). Additionally, saliva contains lysozyme, peroxidase, amylase, secretory leukocyte, lactoferrin, transferrin and antimicrobial proteins (such as salivary immunoglobulins and salivary chaperokine) that are involved in both the innate and acquired immunity, and it represents an important tool for exclusion of transient pathogen microbes entering the mouth. Besides, saliva also plays an important role in the healing of several mucosal lesions, wounds and ulcers (Fábián et al., 2012).

The relevance of oral microbiota in the oral health and the knowledge about its impact on overall human health have been increasing in recent years (Wade, 2013; Arrieta et al., 2014). However, until now most of the studies addressing the oral microbiota had been focused on disease, when the symbiotic balance between the microbiota and the host is lost. This is reflected in the abundant information about the state of oral disease as opposed to the knowledge of the state of the healthy microbiota (Bartold and VanDyke, 2013; Bradshaw and Lynch, 2013; Wade, 2013; Belibasakis, 2014). *In vitro* experiments using dental plaque microcosms indicate that differences in plaque species composition and functional activity are dependent of the individual host environmental characteristics (Filoche et al., 2007; 2008). When the host's diet is rich in easily fermentable carbohydrates, such as sucrose, the pH can fall due to the production of acid by the microbiota, and it could allow colonization by acidophilic species that can result in dental caries (Marsh and Devine, 2011). Moreover, these microbiota responses, biomass yield and pH response to sucrose and starch are also different among individuals, which suggest that the increase in the prevalence of caries-associated pathogens is host specific (Marsh et al., 2011).

I.1.3.2 Intestinal microbiota

The human intestinal microbiota is currently being described as another organ due to the variety of functions that exert (Doré et al., 2013). It plays a role in metabolism, immunity, inflammation and cell proliferation, and is capable of communicating with distant organs of the host, including the brain. A balanced intestinal microbiota is crucial to health; thus, it is considered as a positive attribute, while imbalances or dysbiosis in the intestinal microbiota are associated with development of several diseases (Doré et al., 2013).

In order to summarize the importance of the intestinal microbiota and its activity in the human health, we may classify its functions in metabolic and trophic, protective and immunomodulatory functions (Guarner and Malagelada, 2003; Panda et al., 2014) (Figure I.3).

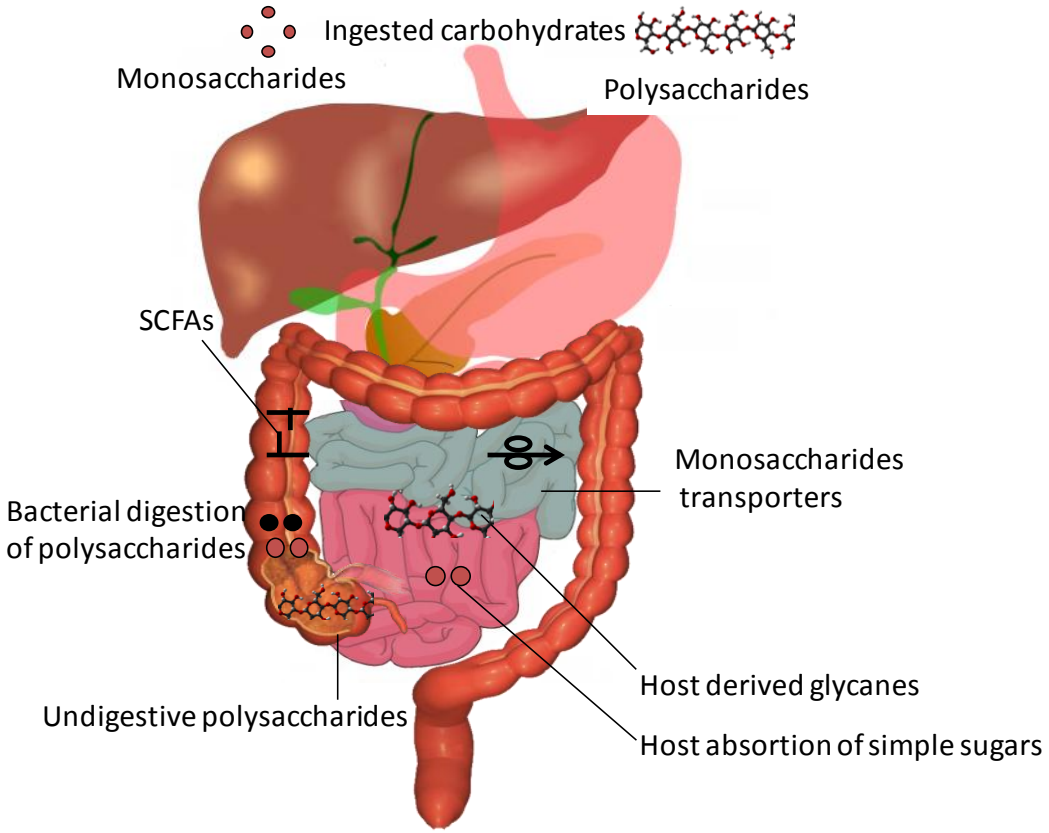
The metabolic and trophic functions of the microbiota provide the host with metabolic capabilities lacking in human intestinal cells such as fermentation of non-digestible dietary components, methanogenesis, gluconeogenesis, fat storage regulation, processing of xenobiotics and biosynthesis of essential amino acids, vitamins and isoprenoids (Walter et al., 2006; Pandeya et al., 2012). Non-digestible carbohydrates present in the colon comprise unabsorbed monosaccharides (fructose, glucose), oligosaccharides and polysaccharides. The intestinal microbiota has the capability to degrade these carbohydrates as its energy source (Flint et al., 2012) (Figure I.2). The main products of this fermentative metabolism are SCFAs, but also it produces lactate, pyruvate, ethanol, succinate as well as the gases H_2, CO_2, CH_4 and H_2S. Among SCFAs, acetate, propionate and butyrate are the most abundant. These products are not only substrates for other colon bacteria but when absorbed in the colon they provide the host with around 10% of the energy requirement (Flint et al., 2012). The SCFAs production also affects the expression of host peptides and hormones, influencing appetite regulation (Sleeth et al., 2010). Propionate has been shown to increase satiety and improve glucose homeostasis. The effects of propionate in the colon epithelium are less known, but due to its absorption and high concentrations in the blood circulation, it is thought to reduce lipogenesis, serum cholesterol levels and carcinogenesis in peripheral tissues (Hosseini et al., 2011). Several beneficial effects have been attributed to butyrate, such as anti-inflammatory and anti-carcinogenic properties (Louis and Flint, 2009; Canani et al., 2010). Oral administration of butyrate to mice fed with a high-fat diet prevented development of insulin resistance and obesity, as reported by Gao and colleagues (2009). This effect was not due to a

reduced food intake, but to an increase in energy expenditure and induction of mitochondria function (Gao et al., 2009). Butyrate has also a trophic effect on the mucosa, it is an important energy source for the colonic epithelium and regulates cell growth, differentiation and renewal of epithelial cells (Cummings, 1995; Hamer et al., 2008; Vital et al., 2014). Due to the intestinal barrier features of the epithelial cells, this trophic function also contributes to the microbiota protective effects to the host (Figure I.3). Acetate, which is known as the major product of intestinal microbial fermentation, can be consumed by many intestinal bacteria giving rise to butyrate (Duncan et al., 2004). This cross-feeding interaction involves not only the butyrate-producing bacteria consuming acetate, but species that convert lactate to butyrate or propionate as well (Flint et al., 2012; Flint et al., 2015) (Figure I.2). The most abundant intestinal butyrate producing bacteria are Firmicutes from *Clostridium* clusters XIVa (such as *Roseburia* and *Eubacterium rectale*) and *Clostridium* clusters IV (such as *Faecalibacterium prausnitzii*) (Louis and Flint, 2009; Vallés et al., 2014; Voreades et al., 2014). Other starch degrading bacteria, such as *Ruminococcus bromii* (*Clostridium* cluster IV) or some *Bifidobacterium* species, can indirectly give rise to a high concentration of butyrate thanks to this bacterial cross-feeding (Leitch et al., 2007; Chassard et al., 2008; Russell et al., 2011).

Increase in SCFAs concentration reduces pH inside the colon and facilitates absorption of other ions. They have been shown to improve the solubility of calcium and to enhance expression of calcium-binding proteins, with the associated bone benefit and the reduction of total cholesterol levels (Flint et al., 2012). The decrease in pH has also important consequences for the composition of the microbiota, for example, *Bacteroides* populations are reduced while butyrate-producing Firmicutes are favoured at mildly acidic pH (Duncan et al., 2009).

In addition, it has been shown that members of the intestinal microbiota such as some lactic acid bacteria (LAB) and *Clostridium* are able to synthesize vitamin K as well as most of the water-soluble B vitamins, such as biotin, cobalamin, folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin and thiamine (LeBlanc et al., 2013).

The concentrations of urea, ammonia and free amino acids available in the ileum are low; nevertheless, large amount of ammonia is produced by colonic proteolytic bacteria from amino acids released from proteins and peptides. These amino acids cannot be absorbed to a significant extent by the colonic epithelium, but are precursors for the synthesis of organic acids by the microbial metabolism, that serve as energy substrates for the colonic mucosa and several peripheral tissues (Davila et al., 2013). However, some adverse effects of the gut microbiota metabolism are associated to an excessive proteolysis inside the colon. The production of detrimental metabolites, such as sulphide and ammonia in high concentration, or other toxic products, can affect the energy metabolism of colonic epithelial cells and have adverse consequences, including carcinogenesis, liver damage, intestinal infections and diarrhoea or constipation (Wallace et al., 2011).



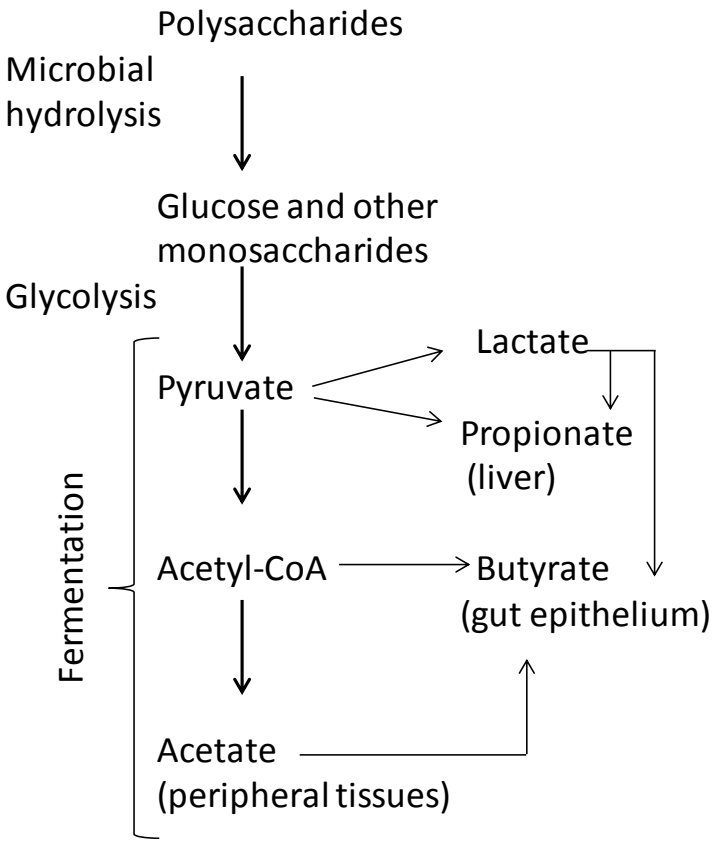


Figure I.2. Microbial carbohydrate metabolism: polysaccharides breakdown.

The protective function of the intestinal microbiota comprises the contribution of the barrier effect and growth inhibition of potential pathogens. In this regard, the microbiota is involved in the production of antimicrobial compounds, such as bacteriocins (Sekirov et al., 2010). Furthermore, it prevents colonization of potentially pathogenic microorganisms by competing for nutrients and epithelial sites and by coaggreation with pathogens (Figure I.3).

Another important activity of the microbiota is that of modulating the immune response (Hooper and Macpherson, 2010; Jarchum and Pamer, 2011; Mazmanian and Lee, 2014). From birth, reciprocal interactions between the microbiota and the mucosal immune response lead to the activation and maturation of the innate and adaptive immune system (Doré et al., 2013). Earliest comparison studies performed on germ-free and colonized mice have revealed a deep effect of microbial colonization on the formation of lymphoid tissues and development of epithelial functions (Hooper et al., 2012). It has been described that epithelial Toll-like receptors are stimulated by intestinal microorganisms limiting the invasion and dissemination of several pathogen bacteria such as *Salmonella enterica* serovar Typhimurium (Vaishnava et al., 2008). Intestinal microbiota has also showed to serve as molecular adjuvant during parasitic infection with *Toxoplasma gondii* by activating cytokine production by dendritic cells (Benson et al., 2009).

The constant challenge with bacterial antigens is crucial for the normal development and function of the gut associated lymphoid tissue (GALT) (Round and Mazmanian, 2009). This includes Peyer's patches, IgA and $CD4^+$ T cells. IgA specific for intestinal bacteria is produced with the aid of dendritic cells, which sample bacteria at the apical surfaces of epithelial cells, allowing monitoring bacteria that are associated with the mucosal surface (Hooper and Macpherson, 2010). Intestinal $CD4^+$ T cells are essential mediators of immune homeostasis and inflammation (Shale

et al., 2013), being pivotal elements in the fact that the presence of commensal microorganisms in the intestine does not produce inflammatory responses, as occurs with the presence of pathogens (Hooper and Macpherson, 2010). When this balance is perturbed, inflammation results, as typified in humans by inflammatory bowel disease, ulcerative colitis and Crohn's disease (Maloy and Powrie, 2011). Moreover, it has been detected alterations of the intestinal microbial composition in patients with these inflammatory diseases (Sokol et al., 2008).

Some other immunological diseases such as allergies and atopic dermatitis have been associated to altered microbiota composition (Watanabe et al., 2003; Hanski et al., 2012). It has been hypothesized that the prevalence of these diseases in people of developed countries has a direct link with decreased contact with natural environmental biodiversity, which has also shown to affect the microbiota composition (Okada et al., 2010; Rook, 2012). Moreover, it has been described that diminished exposure to microbial biodiversity in early life leads to defective immunoregulation, exaggerated cytokine response to social stressors and susceptibility to depression (Rook et al., 2013).

Furthermore, several studies have proposed that there is a pathway connecting the gastrointestinal tract to the brain via hormones and nervus vagus. It is suggested that the gastrointestinal microbiota reports to the brain, affecting mood, behaviour and the corresponding neurochemical changes (Tsurugizawa et al., 2009; Neufeld et al., 2011; Foster and Neufeld, 2013) (Figure I.3). Changes in the microbiota composition in mice have shown to influence behaviour and brain chemistry independently of the autonomic nervous system (Bercik et al., 2011).

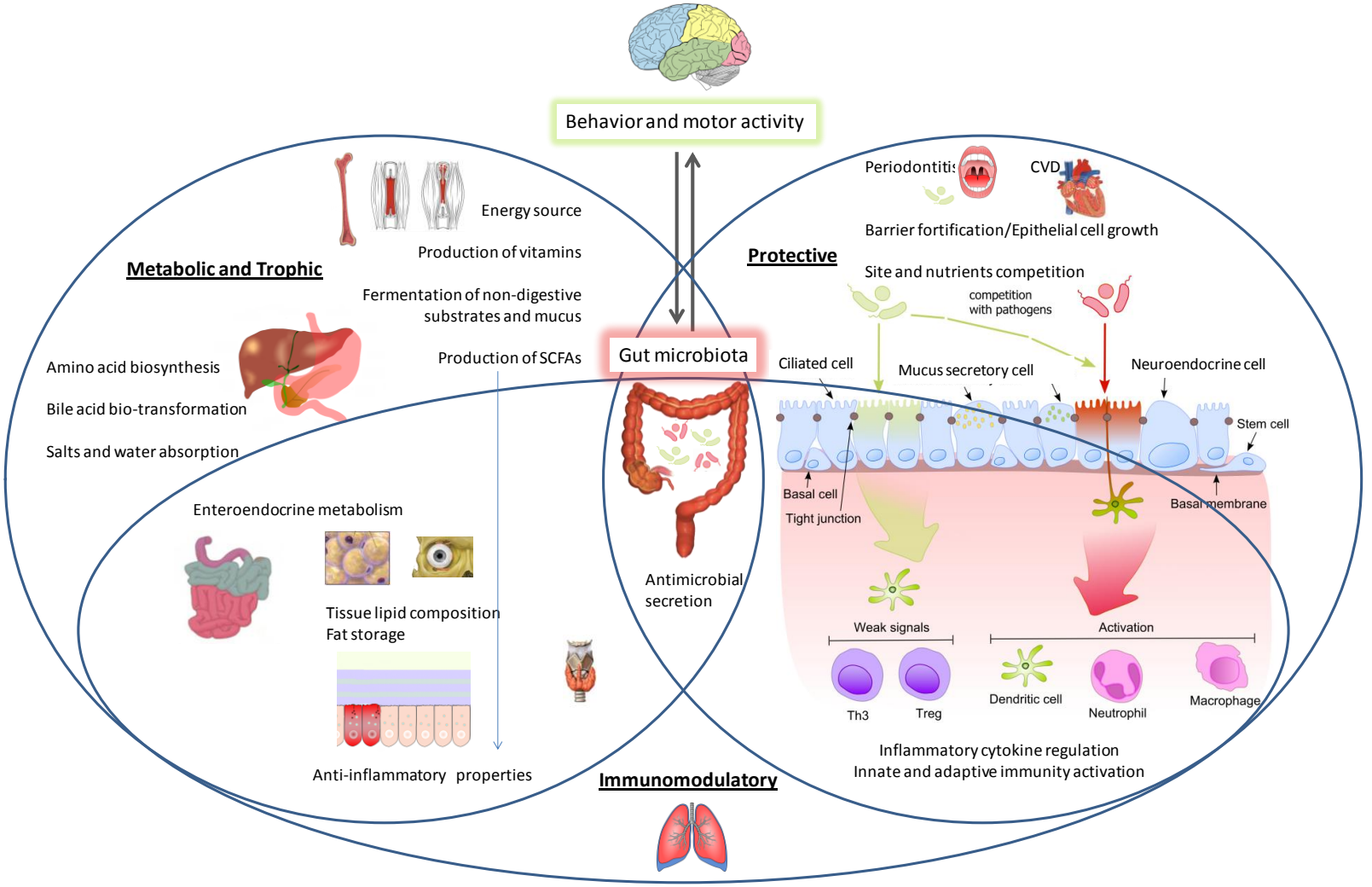


Figure I.3. Functions of the intestinal microbiota.

I.2 ORAL AND INTESTINAL MICROBIOTA MODULATION

The type and number of organisms living in the community depend on the type and quantity of nutrients available and the physicochemical factors in a particular site. The nutritional and physicochemical conditions controlling microbial growth in an ecosystem obey two fundamental laws: Shelford's law of tolerance and Liebig's law of the minimum.

I.2.1 Mechanical and physicochemical factors

According to Shelford's law of tolerance, that involves the non-nutritional factors of an ecosystem, each organism, in order to grow, requires a complex set of physicochemical conditions that have to remain within its tolerance range. Furthermore, microorganisms have to survive any adverse elements, including the innate and acquired immune systems of the host, the production of antimicrobial compounds by the resident microbiota itself (Hooper and Macpherson, 2010; Lakshminarayanan et al., 2013) and the mechanical and hydrodynamic forces present in the site (Wilson, 2005).

In the oral cavity, the temperature (35-36 °C), pH (6-7.5) and oxygen content range (65-10 mm Hg) allow the growth of an extensive range of aerobic and anaerobic microorganisms. Moreover, the mechanical forces generated during chewing and talking cause the detachment of microorganisms that travelled in the saliva (Wilson, 2005).

After the exposure to the low pH in the stomach (from 1.5 to 5, during 1-4 hours), microorganisms are exposed to the effect of bile salts and pancreatic juices at the proximal part of the small intestine (pH 7 during 2-6 hours). In contrast, the lower digestive tract, comprising the terminal ileum and the colon, is characterized by a longer retention time (48–70 hours), allowing dense microbial colonization (Wilson, 2005). At

this point, the microbial activity constantly shapes the colonic environment (Figure I.4). In addition, there is an oxygen gradient from the mucosal surface to the luminal part, where prevail anaerobic conditions. This fact allows that both aerobic and anaerobic microorganisms inhabit the human intestine (Van den Abbeele et al., 2011).

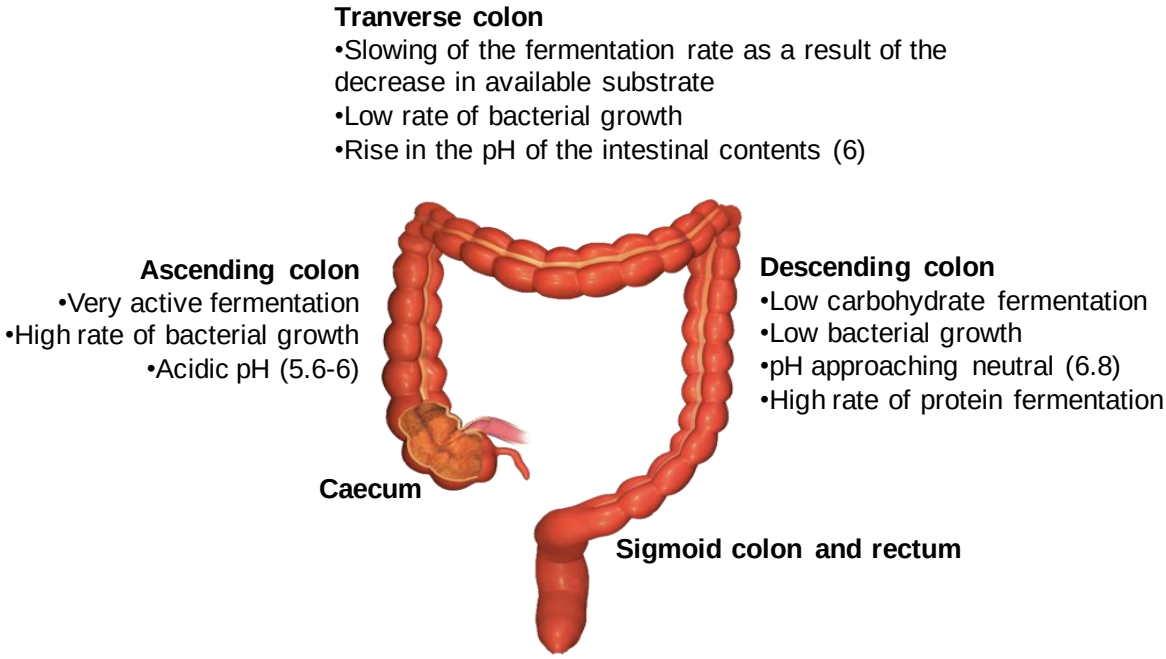


Figure I.4. Microbiological and ecological features inside the colon.

I.2.2 Diet

Liebig's law of the minimum states that the nutrient present on the lower concentration in comparison with the requirements of an organism determines the biomass and yield of this organism. In the oral cavity the main source of nutrients for microbes is saliva, whose principal components are proteins and glycoproteins (such as mucins produced by epithelial cells), but also secreted products of the co-habitant microbes and constituents of dead microbes and epithelial cells which are continously being shed. Furthermore, oral microorganisms are able to obtain nutrients (mainly easily fermentable carbohydrates) from the host's diet, which can impact on the composition of the oral communities (Wilson, 2005).

On the other hand, the species composition of the intestinal microbiota has been shown to respond to dietary changes, determined by competition for substrates and by tolerance of intestinal conditions (Flint et al., 2015). There are a lot of nutrients available for the microbiota in the intestinal tract, such as host secretions, shed cells and bacterial products. However, the principal sources are derived from the host's diet. As stated above, the main nutrients for microbes in the caecum and ascending colon are non-digestive carbohydrates, while in the transverse and descending colon dietary proteins become gradually more important (Wilson, 2005). Undigested fats are not used by colonic bacteria and thus are excreted in the faeces. Mucins produced by the host have an important role in intestinal health, as they form a protective layer on the intestinal epithelium (Derrien et al., 2004). Mucins also represent an energy source for intestinal bacteria (Chassard et al., 2008) including certain *Clostridium* cluster XIVa, *Bacteroides vulgatus*, several *Bifidobacterium* (*B. adolescentis*, *B. breve* and *B. longum*) and *Akkermansia muciniphila* (Derrien et al., 2004; Leitch et al., 2007).

In recent years several authors have described many examples of how changes in diet can affect the microbial populations than inhabit the human colon. Thus, De Filippo and colleagues have observed that, after introduction of solid food, African children, who follow high fibre content diet, had an enrichment of Bacteroidetes and depletion in Firmicutes when compared with European children, who follow a 'western' diet. Indeed they found in African children dominance of *Prevotella* and *Xylanibacter*, bacterial genera known to contain genes for complex plant polysaccharides (cellulose and xylan) hydrolysis, completely lacking in the European children (De Filippo et al., 2010). Similar observation was described by Wu an colleagues that associated *Prevotella* enterotype to people with high-fibre and high-carbohydrate diets, whereas a typical 'western' diet, high in protein and fat, was associated with *Bacteroides* enterotype (Wu et al., 2011). Among the starch-utilizing bacteria, *Roseburia* and *E. rectale* (*Clostridium* cluster XIVa), have showed to diminish when consuming a diet low in carbohydrates content, which has also been associated to a decrease in faecal butyrate. Under these circumstances, *F. prausnitzii* (*Clostridium* cluster IV) became the main butyrate producer bacteria detected (Russell et al., 2011). Additionally, alterations of fibre and fat/protein content in the diets of a small cohort of children and adults caused changes in the microbial composition within a 24-hours period (Wu et al., 2011). It has also been shown that short-term consumption of diets consisting entirely of animal or plant products altered the microbial community structure (David et al., 2014). However, long term monitoring of healthy unrelated adults showed recently that environmentally introduced changes of the intestinal microbiota including dietary regimes throughout adulhood, are primarily affecting the abundance but not the presence of specific microbial species (Rajilić-Stojanović et al., 2013). Furthermore, long term microbial stability in adults has also been demonstrated with 37 US individuals sampled for up to five years (Faith et al., 2013).

In line with the microbial modulatory effect of diet and giving the importance of microbe-host interactions on health and disease, there are scientific, medical and commercial interests in improving microbiota modulation strategies beneficial to the host. There is growing evidence that functional ingredients such as probiotics, prebiotics and symbiotics exert health-promoting effects on the composition, interactions and activities of the intestinal microbiota (Preidis and Versalovic, 2009; Hardy et al., 2013; Kumar et al., 2015), therefore they serve as therapeutic adjuvants to counteract balance disruptions of the microbiota occurring in several diseases.

In recent years, new dietary approaches to modulate the microbiota are being investigated. In this regard dietary polyphenols, present in tea, cranberry, grapes or red wine among other foods, have been identified as potential antimicrobial agents with bacteriostatic or bactericidal actions (Duda-Chodak, 2012). They have been proposed as anticariogenic components of mouthwashes or toothpastes (Navarrete and Rizzardinni, 2011) and their intestinal microbiota modulatory properties are also starting to be studied (Etxcheberria et al., 2013).

I.2.2.1 Probiotics

The interest in the role that the human intestinal microbiota has in disease has continuously increased since the 1900s, when Metchnikoff proposed the concept that the intestinal microbiota can be modulated by the administration of bacteria. He established the hypothesis that replacing or diminishing putrefactive bacteria in the intestine with LAB could normalize bowel health and prolong life (Metchnikoff and Mitchell, 1907). After many years, probiotics were defined as ‘‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’’ (FAO/WHO, 2002). Recently, it has been suggested that probiotics do not need to be alive, because their cellular components,

such as cell wall peptidoglycans, could be sufficient to induce benefits (Collins, 2014). The International Scientific Association for Probiotics and Prebiotics (ISAPP) has recently elaborated consensus recommendations in order to take into account advances in science and applications and to unify criteria (Hill et al., 2014). They have also included in the concept of probiotics new commensals and consortia of defined strains from human samples with adequate evidence of safety and efficacy (Hill et al., 2014).

Several important health-promoting characteristics can be observed in some microorganisms that are considered as probiotics, these are: (1) transient modulation of the gut microbial composition, (2) exclusion or inhibition of pathogens in the gut, (3) enhancement or maintenance of the epithelial barrier and (4) modulation of the immune system (Collado et al., 2009; Lebeer et al., 2010). The majority of probiotic bacteria are bifidobacteria and LAB, mainly *Lactobacillus*, although *Lactococcus*, *Streptococcus* and *Enterococcus* species, certain yeast strains and some non-pathogenic strains of *Escherichia coli*, are also recognized as probiotics (Huycke and Gaskins, 2004; Dogi et al., 2008). Most evidence available about the impact of probiotic microorganisms on the microbiota composition and functions has been obtained by targeting *Lactobacillus* and *Bifidobacterium* (Savard et al., 2011), although the use of probiotics could also have an impact on other bacterial genera relevant to the intestinal health. Probiotics have confirmed their potential as therapeutic options for a variety of diseases with clinically documented health effects in human subjects (Strobel and Mowat, 2006), such as diarrhoea reduction in children (Deshpande et al., 2010; Kale-Pradhan et al., 2010; Johnston et al., 2011) or prevention of allergic diseases, atopic eczema and rhinoconjunctivitis (Ouwehand et al., 2009; Bertelsen et al., 2014), among others.

The beneficial properties of probiotic bacteria depend on the specific characteristics of a particular species and can vary even between

closely related bacterial strains. One example is the species *Lactobacillus plantarum* whose different strains can be found or isolated from healthy human colonic mucosa, human saliva, green olives, meat, chucrut or dairy products and exert different activities depending on the specific strain. It has been described an antagonistic effect against *Helicobacter pylori* caused by *L. plantarum* MLBPL1 (Rokka et al., 2006) and the reduction of enterocolitis in rats assisted by *L. plantarum* DSM 9843 (Mao et al., 1996). *L. plantarum* strain b240 showed a strong antagonistic effect against *S. enterica* serovar Typhimurium on intestinal epithelial cells, probably due to a high ability to induce IgA (Ishikawa et al., 2010). Goossens and colleagues observed an increase in the intestinal content of LAB and clostridia by the intake of *L. plantarum* 299v, in patients with previous history of polyps or a positive family history of colorectal cancer (Goossens et al., 2006). More recently, *L. plantarum* DK119, isolated from fermented Korean cabbage, has showed antiviral effects on influenza virus infection by modulating the host innate immunity of dendritic and macrophage cells and the cytokine production pattern in mice (Park et al., 2013). Additionally, other *L. plantarum* strains (Lp09 and Lp45) have been explored as probiotic agents for the management of hypercholesterolemia in rats, with promising results (Huang et al., 2013).

Our research group has focused on the potential probiotic strain *L. plantarum* IFPL935, isolated from a raw goat's milk cheese (Fontecha et al., 1990). In previous studies, it has been reported that *L. plantarum* IFPL935 was able to initiate the catabolic pathway of flavan-3-ols present in a grape seed extract (Tabasco et al., 2011). Adhesion to the intestinal mucosa is regarded as a prerequisite for colonization and is an important characteristic related to the ability of probiotic strains to confer a health benefit by stimulation of the host immune system and competition with pathogens. In this regard, *L. plantarum* IFP935 has shown the capacity of adhere to epithelial cell lines (Caco-2 and HT-29) (Bustos et al., 2012). Furthermore, *in vitro* studies have demonstrated that *L. plantarum*

IFPL935 inhibited significantly the adhesion to epithelial cells of the pathogenic strain *Staphylococcus aureus* CECT240 (García-Cayuela et al., 2014).

I.2.2.2 Prebiotics

A prebiotic was first defined by Gibson and Roberfroid in 1995 as a 'non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health' (Gibson and Roberfroid, 1995). Later on time, the same authors updated this definition in order to extend the concept to the entire intestinal tract (Gibson et al., 2004). Then, in 2008, the ISAPP has defined 'a dietary prebiotic' as, 'a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health'.

Several oligosaccharides and polysaccharides (including dietary fibre) have been claimed to have a prebiotic effect, but not all dietary carbohydrates are prebiotics. In order, for a food ingredient, to be considered as a prebiotic, it has to obey three criteria: (1) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption, (2) fermentation by intestinal microbiota and (3) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing.

The demonstration of the prebiotic effect of these food components has to be carried out *in vitro* and *in vivo* by the performance of nutritional feeding trials in the targeted species, as occurs with other functional foods (Diplock et al., 1999). The prebiotics commonly used in the food industry are short-chain oligosaccharides, which are easily fermented in the ascending colon, where the microbiota is known to have a saccharolytic metabolism (Macfarlane et al., 1992) (Figure I.2). As a

consequence, the distal colon is dominated by a proteolytic metabolism, leading to the formation of polyamines and ammonia, which are regarded as a risk factor for chronic colonic diseases (such as colon carcinoma) (Cassidy et al., 1994; Terpend et al., 2013) (Figure I.2). Consequently, there is a great interest in finding prebiotics that exert biological activity in the distal colon (Terpend et al., 2013). In order to do that, it is useful to combine short-chain with long-chain fructans (Van Loo, 2004). In this line, it has been described the enzymatic synthesis of oligosaccharides derived from lactulose (OsLu) aiming at the production of a new group of more slowly fermenting prebiotics (Cardelle-Cobas et al., 2008; Cardelle-Cobas et al., 2012).

The most tested examples of prebiotic compounds are inulin-type fructans (ITF) and galacto-oligosaccharides (GOS) (Gibson and Roberfroid, 2008). However, there are a variety of fermentable ingredients, such as fructo-oligosaccharides (FOS), gluco-oligosaccharides, lactulose, isomalto-oligosaccharides, soy oligosaccharides, pectins, xylooligosaccharides (XOS) and various gums and mucopolysaccharides that have also demonstrated their prebiotic potential (Roberfroid et al., 2010). These components are naturally found in some fruits, vegetables and grains. There is evidence that dietary supplementation with FOS and inulin can promote specific groups of bacteria (Bouhnik et al., 2004; Ramirez-Farias et al., 2009). Today, the prebiotic effect is commonly monitored by using bifidobacteria or lactobacilli as markers, but may include other species in the future, if their positive health benefits can be confirmed (Roberfroid et al., 2010). Certain species of *Ruminococcaceae* and *Lachnospiraceae* are recognized to exert potentially beneficial health effects due to the production of butyrate. Some authors have reported different selective growth profiles of these bacteria on several prebiotic substrates, which included starch, inulin, FOS, GOS and XOS (Scott et al., 2014).

Infant formula/foods have been supplemented with bifidogenic prebiotics in order to mimic the naturally presence of HMOs from mother's milk and prevent possible paediatric diseases (Bakker-Zierikzee et al., 2006; Scholtens et al., 2008). Some preliminary studies have shown promising results of the use of prebiotics by inducing changes in intestinal microbiota composition of patients with inflammatory bowel disease (IBS) that improve their intestinal activity and well-being (Paineau et al., 2008). Several experimental studies with prebiotics have been focused on the prevention of colon cancer due to the role of the intestinal microbiota composition in the reduction of toxic substances inside the colon (Rowland et al., 2009). It has been also described the intake of certain foods with prebiotic properties as a good strategy to prevent obesity-related disorders (Cani et al., 2009).

Mixtures of both probiotics and prebiotics are referred to as symbiotics. The interest of these products is due to the selective effect that the prebiotic could promote on the probiotic component. This could enhance the viability of the probiotic bacteria during storage and during the passage through the gastrointestinal tract and promote growing of this specific probiotic bacteria (Marx et al., 2000; Schrezenmeir and De Vrese, 2001; Champagne et al., 2005). Certain symbiotics have shown to be more effective than probiotics or prebiotics alone in colorectal cancer prevention (Liong et al., 2008), in improving the quality of life in patients suffering from ulcerative colitis (Fujimori et al., 2009) or in general modulation of the microbiota (Saulnier et al., 2008; Grimoud et al., 2010).

I.2.2.3 Polyphenols

Polyphenols are plant-derived compounds that show an extensive variety of chemical structures. They are classified as flavonoids and nonflavonoids. Polyphenols are one of the most diverse bioactive phytochemicals which can be found in a wide range of foods such as

onions, tea, cacao, strawberries, cranberries, grapes, wine, etc. There are evidences of a dual interaction of microbiota and these food components. Thus, polyphenols can modulate the microbiota, inhibiting the development of some bacterial groups, while the microbiota can transform these food compounds into different metabolites based on the different microbiota functionalities (Lee et al., 2006; Tzounis et al., 2008; Kemperman et al., 2013). These metabolites are often better absorbed than the parent food phenolics, and could have specific biological effects that extend those of their precursors (Selma et al., 2009). It has been estimated that 90–95% of dietary polyphenols are not absorbed in the small intestine and therefore accumulate in the colon (Tuohy et al., 2012). In this regard, through microbial transformations, polyphenols could be absorbed and reach the target tissues and organs in order to exert its biological effect. Therefore, potential positive effects of polyphenol-rich foods might be dependent on biotransformation into more bioavailable forms by intestinal bacteria, potentially modulating related health benefits.

There is increasing evidence that health benefits associated to the moderate consumption of red wine could be due to its phenolic compounds. *In vitro* and animal model studies have shown protective effects of red wine polyphenols against atherosclerosis, such as inhibition of LDL oxidation in human plasma (Lourenço et al., 2008) and relaxations of coronary arteries in pigs (Ndiaye et al., 2003). Furthermore, wine polyphenols could exhibit colon cancer inhibition (Franke et al., 2002) and antibacterial effects (Cueva et al., 2010). The interest in the interactions between polyphenols and intestinal microbiota has increased in the last years (Selma et al., 2009; Pozo-Bayón et al., 2012; Espley et al., 2014). Wine and grape derivatives are attractive products for studying the impact on microbiota through polyphenols metabolism due to their high content and structural diversity (Forester and Waterhouse, 2009). Phenolic compounds of wine consist of a complex mixture of flavonoids, such as flavan-3-ols or flavanols and anthocyanins, but also of nonflavonoids such

as resveratrol, cinnamates and gallic acid. The non-glycosylated phenolic compounds can be directly absorbed in the small intestine. However, glycosylated polyphenols including anthocyanins, flavonols and resveratrol glycosides could be hydrolyzed by the action of ß-glucosidases from bacteria and/or epithelial cells in the mouth (Walle et al., 2005) (Figure I.5). Further transformations take place in the colon involving the formation of simple phenols, phenolic and aromatic acids and lactones with different degrees of hydroxylation and side chain length that could be further absorbed and subsequently submitted to intestinal and hepatic metabolism by phase II enzymes (Aura, 2008; Selma et al., 2009) (Figure I.5).

Additionally, metabolic studies have shown changes in the metabolic profile of urine, plasma and faeces, following the ingestion of polyphenol rich foods and beverages, including grape or wine extracts intake (Grün et al., 2008; Jacobs et al., 2008; Bolca et al., 2013). For example, in the case of proanthocyanidins, it has been suggested that the microbial capacity to metabolize these compounds is affected by a predominant microbial inhibitory effect (Aura, 2012).

Several *in vitro* studies have been conducted with the purpose of screen the selective action of different phenolic compounds at different concentrations. Tzounis and colleagues showed that the incubations of faecal microbiota with flavanol monomers (catechin and epicatechin) at different concentrations favour the growth of *B. coccoides-E. rectale* and *Lactobacillus* and *Bifidobacterium* (at a lower concentration). By contrast, at the highest concentration assayed, the growth of *Clostridium histolyticum* was decreased (Tzounis et al., 2008). By means of *in vitro* gastrointestinal simulation techniques and pyrosequencing analysis, it was observed that a red wine-grape extract favoured the growth of *Klebsiella*, *Alistipes* and *Akkermansia*, while *Bifidobacterium*, *B. coccoides* and *Bacteroides* showed a decrease (Kemperman et al.,

2013). In a human intervention study comparing gin, red wine and dealcoholized red wine, a selective effect on microbial modulation was observed in faeces. Regarding red wine intake, the phyla Proteobacteria, Fusobacteria, Firmicutes and Bacteroidetes, and the genera *Bifidobacterium* and *Prevotella* experienced an increase; however, *Clostridium* and in particular *C. histolyticum* showed a decrease during the intervention with red wine. By contrast, these effects disappeared or were minimized after a deacolcoholized red wine intake (Queipo-Ortuño et al., 2012). The different findings on the selective antimicrobial effect of polyphenols and the small number of volunteers tested make necessary to undertake more investigations in this line and open a way in the development of new strategies for studying the potential intestinal microbial modulation by polyphenols.

On the other hand, several *in vitro* and *in vivo* studies have shown that wine components also inhibit the adhesion of and/or exert activity against oral bacteria (Kongstad et al., 2008; Furiga et al., 2009). Comparisons of microbiota profiles from the supra- and subgingival plaques in adults with different drinking habits have shown that wine drinkers present lowest numbers of different microbial groups than water drinkers (Signoretto et al., 2010). As a potential accessory for the control of periodontal disease, wine catechins have shown to exert a strong antimicrobial effect against *Porphyromonas gingivalis* and *Prevotella intermedia* (Ho et al., 2001). Proanthocyanidins from red wine have been recognized as one of the most powerful components with anti-adhesion and anti-biofilm activity against *S. mutans* (Daglia et al., 2010).

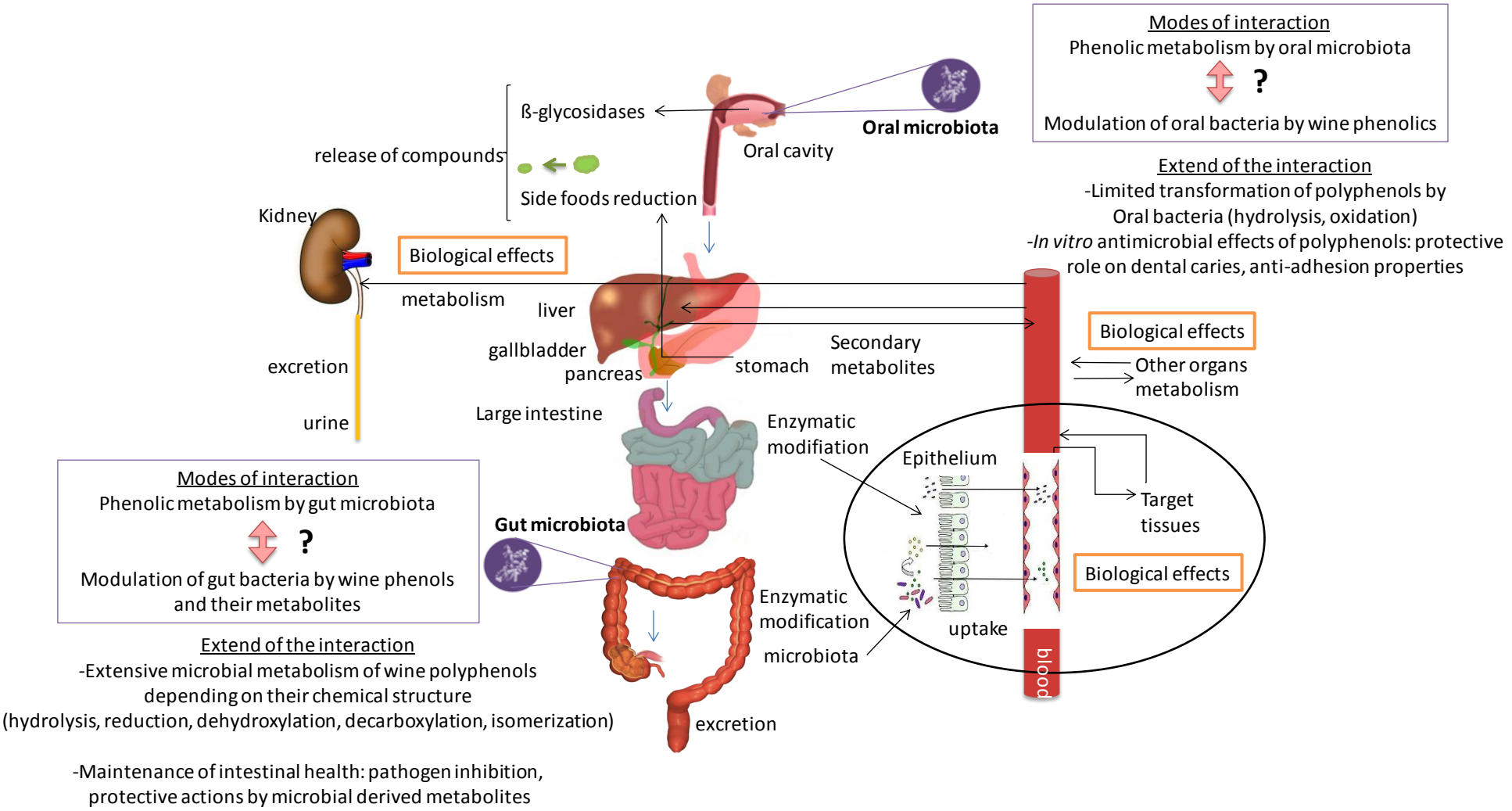


Figure I.5. General scheme of polyphenols metabolism.

I.3 *IN VITRO* MODELS TO STUDY THE HUMAN GUT MICROBIOTA

The evaluation of intestinal microbial diversity by either cultivable or genomic methodologies has been mostly carried out with faecal samples. These samples are considered as representative of the distal large intestine; however, they do not provide knowledge about dynamic microbial process at the site of fermentation in the intestine. All the *in vitro* fermentative models have the common purpose of cultivate a complex intestinal microbiota under controlled environmental conditions for carrying out microbial modulation and metabolism studies. Thus, they are considered excellent tools to study the human gut microbiota, given the difficulty to access the main niches of colonization in the gastrointestinal tract. Moreover, they allow the screening of a large variety of experimental conditions such as dietary ingredients without ethical constraints.

The simplest *in vitro* models to study the intestinal microbiota are static batch fermentations. These models are generally performed in small sealed bottles or reactors with either a range of pure or mixed cultures of strains or suspensions of faecal material containing microbial communities from human origin. They are used to describe microbial growth and their ability to metabolize different substrates. These systems have the inconvenient that changes in pH and the accumulation of microbial metabolites lead to the inhibition of microbial activity, so they could not represent the facts occurring *in vivo*. In spite of these methodological limitations, faecal batch incubations have demonstrated to be a useful tool to investigate metabolic profiles of SCFAs produced by intestinal microbiota from the fermentation of a large variety of dietary components such as inulin-type fructans and resistant starch complex carbohydrates (Pompei et al., 2008; Lesmes et al., 2008). They have

been valuable for a first assessment of intestinal polyphenols metabolism (Gross et al., 2010) or for comparison of different sources or doses of polyphenols (Possemiers et al., 2007; Bolca et al., 2009; De Weirdt et al., 2010). The inoculation density, together with the substrate depletion rate, also defines microbial growth in these systems. Low cell densities allow typical S-shaped growth curve due to the presence of an initial abundant nutrient and the consumption of the substrates at the end of the incubation. Conversely, systems with high cell densities similar to those found in the colon result in limited growth (Payne et al., 2012).

In contrast to short-duration experiments with batch models, long–term experiments with dynamic, multi-compartment gastrointestinal simulators are used when gut microbial dynamics and activities need to be assessed. The clear advantage of these models is to allow real time measurement of the effects of foods or other chemical compounds in the gastrointestinal ecosystem (Kong and Singh, 2010; Wickham and Faulks, 2012). Most of these dynamic models are based on the Reading model firstly described by Gibson and colleagues (1988), which involves a three-stage continuous culture system simulating the ascending, transverse and distal colon. This system allowed setting of pH for the three vessels at 5.5, 6.2 and 6.8 respectively, which is a critical parameter for this microbial ecosystem. The nutritious medium commonly used consists of protein substrates (casein and peptone), complex carbohydrates (pectin, xylan, arabinogalactan and resistant starches) that are not digested by gastrointestinal enzymes, and a mixture of salts and vitamins (Gibson et al., 1988). The continuous replenishment of nutrients and the control of physiological temperature and anaerobic conditions are crucial in the adaptation and survival of the *in vitro* gut microbiota. This allows the establishment of steady-state conditions in terms of microbial composition and metabolic activity. Furthermore, the control of defined pH values, downstream nutrient limitations and retention times in the different *in vitro* compartments allows a region-specific differentiation of the microbial

communities and their activity (Van den Abbeele et al., 2010). Anaerobic conditions are usually reached through continuous flushing of CO_2 or N_2. Some examples of these dynamic fermentation models are the SHIME (Simulator of the Human Intestinal Microbial Ecosystem), consisting in three glass reactors that represent the ascending, transverse and descending colon and that possess two previous glass vessels representing the gastric and duodenal stages (Molly et al., 1993), and the TIM-2 (Minekus et al., 1999) composed by a continuous single-stage fermenter to simulate the proximal colon conditions. The TIM-2 is able to reproduce the peristaltic mixing of the luminal content and the absorption of water and fermentation products. The previously designed TIM-1 (Minekus et al. 1995) can simulate the process occurring in the upper gastrointestinal tract (stomach and small intestine) and the effluent of the TIM-1 can be manually introduced in the TIM-2 in order to mimic the full transit through the gastrointestinal system (Hatanaka et al., 2012). Comparisons of results obtained from the TIM-2 and SHIME models have demonstrated the suitability of both models when studying the effects of diet on SCFAs production and its influence on specific bacteria (Van den Abbeele et al., 2013). Another example of fermentation model is the twin-vessel single-stage chemostat model recently described by McDonald and colleagues (2014), which reproduces the human distal gut environment by maintaining neutral pH conditions and a constant culture volume. Moreover, recent developments of a single-stage model simulating the ileum microbiota have been reported (Venema and Van den Abbeele, 2013).

The colonization, reproducibility and functional stability of human gut microbiota inside the models depend in part of the inoculum. They can be used diverse alternatives depending on the purpose of the study. Inoculation with liquid faecal suspension from one individual can be useful for biological repetition of a study in order to observe individual variability or stability (Van den Abbeele et al., 2010). Pooling stools from several

individuals can be used to inoculate a batch fermenter and produce a standardized inoculum to be frozen and reused in order to obtain sets of equivalent inocula (Rajilić-Stojanović et al., 2010; Martinez et al., 2013). Cinquin and colleagues (2004) have developed an immobilization process for the entrapment of faecal microbiota in mixed xanthan-gellan gum gel beads with the purpose of establish biofilm-associated states of microbial populations in conjunction with a continuous wash-out of less competitive bacteria. This last system has recently been updated into the model PolyFermS, allowing maintenance of the microbial diversity over long time and performance of parallel experiments with exactly the same microbiota (Berner et al., 2013). Recent developments focusing in reproducibility of the experiments face the inoculation of the *in vitro* models with defined populations of pure strains from the human gut microbiota (Newton et al., 2013).

Since most of the models have been focused on simulating either the upper gastric-small intestine digestion or the colonic fermentation process, our research group has pursued the development of a new system which combines the gastrointestinal process from the stomach until the final part of the colon under a unique computer controller. This new system, named SIMGI, is composed by a gastric compartment that simulates peristaltic mixing movements, a reactor simulating the small intestine and three stage continuous fermenters that reproduce the colon region-specific microbiota and its metabolism. It possesses the particularity to allow joint or separated simulation of the gastric and colonic fermentative processes (Barroso et al., 2015).

The ability of the *in vitro* gut models described above to simulate the *in vivo* conditions is limited by the lack of the intestinal epithelium and mucus. The evaluation of the host's response is normally conducted by means of cell culture experiments. They are easy and quick ways to study cellular behaviour, cell signalling pathways and cell interactions. The most

frequent cell lines currently used are Caco-2 and HT29-MTX that are able to form a polarized monolayer of differentiated intestinal epithelial cells (Lesuffleur et al., 1991; Louvard et al., 1992; Sambuy et al., 2005). Furthermore, co-cultures of cell lines in combination with immune cells have been characterized in an attempt to mimic the human intestinal mucosal environment (Christoffersen et al., 2012).

Additional tools for modelling the physiological colonic conditions are the incorporation of mucosal environment devices inside the fermentation vessels in order to differentiate between microbial biofilms adhering to the devices and luminal microbiota (Macfarlane et al., 2005; Van den Abbeele et al., 2012). More recently, the Host-Microbiota Interaction (HMI) module has been developed (Marzorati et al., 2014), consisting of two compartment devices separated by a functional double-layer composed of an upper mucus layer and a lower semi-permeable membrane allowing molecules and oxygen transport. In the upper part, a complex microbial community colonises the mucus, simulating the luminal side of the colon while the lower part contains enterocyte human cells (Marzorati et al., 2014). This combination of *in vitro* models closely represents the ideal experimental model described by Fritz and colleages (2013) for studying host-microbiota interactions. They stated that physiological pH, fluid retention times and dissolved oxygen concentrations have to be maintained and that the model should include the mucus layer, epithelial cells and complex gut microbiota in anaerobic/microaerophilic conditions.

Overall, to get the complete picture, combining both *in vitro* and *in vivo* studies could represent an appropriate approach to understand how microbiota interacts with the host. The use of animal models is an excellent tool for this purpose. For example, diet-induced alterations in microbiota composition of zebrafish have shown to influence fat absorption (Semova et al., 2012). Moreover, development of germ-free

mice (Reyniers et al., 1959) has helped to understand the microbiota-host interactions and have allowed highly controlled working conditions. Transplantation of human faecal microbiota into germ-free mice can be viewed as capturing an individual's microbial community at a fixed moment in time (Goodman et al., 2011; Kau et al., 2011; Ridaura et al., 2013).

Nevertheless, clinical trials are required to confirm results obtained through all of these study models. As it has been exemplified above, until date, several human trials have been performed in order to test the microbiota modulatory effects of probiotics (Savard et al., 2011; Johnston et al., 2011), prebiotics (Roberfroid et al., 2010) and other components of the diet such as polyphenols (Etxeberria et al., 2013).

The growing evidence about the important role of the human microbiota in health leads to the development of new dietary ingredients and approaches for modulating the microbiota. Thus, it is necessary to perform both *in vitro* and *in vivo* experiments to deeper understand the dynamic interactions inside the human microbial community and with the host. Further, it remains essential to conduct more studies combining biochemical, microbiological and clinical parameters in order to know the effectiveness of the microbial modulatory strategies and their impact on health.

I.4 REFERENCES

Aagaard, K., Ma, J., Antony, K. M., Ganu, R., Petrosino, J., & Versalovic, J. (2014). The placenta harbors a unique microbiome. Science Translational Medicine, 6, 237ra65.

Arrieta, M. C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M., & Finlay, B. (2014). The intestinal microbiome in early life: health and disease. Frontiers in Immunology, 5, 427.

Arumugam et al. MetaHIT Consortium. (2011). Enterotypes of the human gut microbiome. Nature, 473, 174-180.

Aura, A. M, Mattila, I., Hyötyläinen, T., Gopalacharyulu, P., Cheynier, V., Souquet, J. M., Bes, M., Bourvellec, C., Guyot, S., & Orešič, M. (2012). Characterization of microbial metabolism of Syrah grape products in an *in vitro* colon model using targeted and non-targeted analytical approaches. European Journal of Nutrition, 52, 833-846.

Aura, A. M. (2008). Microbial metabolism of dietary phenolic compounds. Phytochemistry Reviews, 7, 407-429.

Bäckhed, F., Ley R. E., Sonnenburg, J. L., Peterson, D.A., & Gordon, J. I. (2005). Host bacterial mutualism in the human intestine. Science, 307, 1915–1920.

Bakker-Zierikzee, A. M., Tol, E. V., Kroes, H., Alles, M. S., Kok, F. J., & Bindels, J. G. (2006). Faecal SIgA secretion in infants fed on pre-or probiotic infant formula. Pediatric Allergy and Immunology, 17, 134-140.

Barroso E., Cueva C., Peláez C., Martínez-Cuesta M. C., Requena T. (2015). Development of human colonic microbiota in the computer-controlled dynamic SIMulator of the GastroIntestinal tract SIMGI. LWT Journal of Food Science and Technology, 61, 283-289.

Bartold, P. M., & Van Dyke, T. E. (2013). Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts. Periodontology 2000, 62, 203-217.

Belibasakis, G. N. (2014).Microbiological and immuno-pathological aspects of peri-implant diseases. Archives of Oral Biology, 59, 66-72.

Benson, A., Pifer, R., Behrendt, C. L., Hooper, L. V., & Yarovinsky, F. (2009). Gut commensal bacteria direct a protective immune response against *Toxoplasma gondii*. Cell Host & Microbe, 6, 187-196.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y., Blennerhassett P., Macri, J., McCoy K.D., Verdu E. F., & M. Collins S.M. (2011). The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology, 141, 599-609.

Berner, A. Z., Fuentes S., Dostal, A., Payne, A. N., Gutierrez, P. V., Chassard, C., Grattepanche, F., de Vos, W. M. & Lacroix, C. (2013). Novel Polyfermentor Intestinal Model (PolyFermS) for controlled ecological studies: validation and effect of pH. PloS One, 8, e77772.

Bertelsen, R. J., Brantsæter, A. L., Magnus, M. C., Haugen, M., Myhre, R., Jacobsson, B., Longnecker, M. P., Meltzer H. M., & London, S. J. (2014). Probiotic milk consumption in pregnancy and infancy and subsequent childhood allergic diseases. Journal of Allergy and Clinical Immunology, 133, 165-171.

Bezirtzoglou, E., Tsiotsias, A., & Welling, G. W. (2011). Microbiota profile in feces of breast-and formula-fed newborns by using fluorescence in situ hybridization (FISH). Anaerobe, 17, 478-482.

Bolca, S., Van de Wiele, T., Possemiers, S. (2013). Gut metabotypes govern health effects of dietary polyphenols. Current Opinion in Biotechnology, 24, 220-225.

Bolca, S., Wyns, C., Possemiers, S., Depypere, H., De Keukeleire, D., Bracke, M, Verstraete, W. & Heyerick, A. (2009). Cosupplementation of isoflavones, prenylflavonoids, and lignans alters human exposure to phytoestrogen-derived 17β-estradiol equivalents. The Journal of Nutrition, 139, 2293-2300.

Bouhnik, Y., Raskine, L., Simoneau, G., Vicaut, E., Neut, C., Flourié, B, Brouns F. & Bornet, F. R. (2004). The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. The American Journal of Clinical Nutrition, 80, 1658-1664.

Bradshaw, D. J., & Lynch, R. J. (2013). Diet and the microbial aetiology of dental caries: new paradigms. International Dental Journal, 63, 64-72.

Bustos I., García-Cayuela T., Hernández-Ledesma B., Peláez C., Requena T., Martínez-Cuesta M. C. (2012). Effect of flavan-3-ols on the adhesion of potential probiotics lactobacilli to intestinal cells. Journal of Agricultural and Food Chemistry, 60, 9082–9088.

Callewaert, C., Kerckhof, F. M., Granitsiotis, M. S., Van Gele, M., Van de Wiele, T., & Boon, N. (2013). Characterization of *Staphylococcus* and *Corynebacterium* clusters in the human axillary region. PloS One, 8, e70538.

Canani, R. B., Costanzo, M. D., Leone, L., Pedata, M., Meli, R., & Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World Journal of Gastroenterology, 17, 1519-1528.

Cani, P. D., Lecourt, E., Dewulf, E.M., Florence, M. Sohet, F. M., Pachikian, B. D., Naslain, D., De Backer, F., Neyrinck, A. M., & Delzenne,

N. M (2009) Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. The American Journal of Clinical Nutrition, 90, 1236-1243.

Cardelle-Cobas A., Martínez-Villaluenga C., Villamiel M., Olano A., & Corzo N. (2008). Synthesis of oligosaccharides derived from lactulose and pectinex ultra SP-L. Journal of Agricultural and Food Chemistry, 56, 3328-3333.

Cardelle-Cobas, A., Olano, A., Corzo, N., Villamiel, M., Collins, M., Kolida, S., & Rastall, R. A. (2012). *In vitro* fermentation of lactulose-derived oligosaccharides by mixed fecal microbiota. Journal of Agricultural and Food Chemistry, 60, 2024-2032.

Cassidy, A., Bingham, S. A., & Cummings, J. H. (1994). Starch intake and colorectal cancer risk: an international comparison. British Journal of Cancer, 69, 937.

Cervera-Tison, M., Tailford, L. E., Fuell, C., Bruel, L., Sulzenbacher, G., Henrissat, B., Berrin, J. G., Fons, M., Giardina, T.& Juge, N. (2012). Functional analysis of family GH36 α-galactosidases from *Ruminococcus gnavus* E1: insights into the metabolism of a plant oligosaccharide by a human gut symbiont. Applied and Environmental Microbiology, 78, 7720-7732.

Shale, M., Schiering, C., & Powrie, F. (2013). $CD4^+$ T-cell subsets in intestinal inflammation. Immunological Reviews, 252, 164-182.

Champagne, C.P., & Gardner, N.J. (2005). Challenges in the addition of probiotic cultures to foods. Critical Reviews in Food Science and Nutrition, 45, 61-84.

Charlson, E. S., Bittinger, K., Haas, A. R., Fitzgerald, A. S., Frank, I., Yadav, A., Bushman, F. D. & Collman, R. G. (2011). Topographical

continuity of bacterial populations in the healthy human respiratory tract. American Journal of Respiratory and Critical Care Medicine, 184, 957-963.

Chassard, C., Scott, K.P., Marquet, P., Martin, J.C., Del'homme, C., Dapoigny, M., Flint, H.J. & Bernalier-Donadille A. (2008). Assessment of metabolic diversity within the intestinal microbiota from healthy humans using combined molecular and cultural approaches. FEMS Microbiology Ecology, 66, 496-504.

Christoffersen, T. E., Jensen, H., Kleiveland, C. R., Dørum, G., Jacobsen, M., & Lea, T. (2012). *In vitro* comparison of commensal, probiotic and pathogenic strains of *Enterococcus faecalis*. British Journal of Nutrition, 108, 2043-2053.

Cinquin, C., Le Blay, G., Fliss, I. & Lacroix, C. (2004). Immobilization of infant fecal microbiota and utilization in an *in vitro* colonic fermentation model. Microbial Ecology, 48, 128-138.

Claesson, M.J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., Marchesi, J.R., Falush, D., Dinan, T., Fitzgerald, G., Shanahan, F., Twomey, C., Hill, C., Ross, R. P. & O'Toole, P. W. (2011). Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proceedings of the National Academy of Sciences of the United States of America, 108, 4586-4591.

Collado, M.C., Isolauri, E., Salminen, S., & Sanz, Y. (2009). The impact of probiotic on gut health. Current Drug Metabolism, 10, 68-78.

Collins, S. M. (2014). A role for the gut microbiota in IBS. Nature reviews Gastroenterology & Hepatology, 11, 497-505.

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial community variation in human body habitats across space and time. Science, 326, 1694-1697.

Cueva, C.,Moreno-Arribas, M. V.,Martin-Alvarez, P.J., Bills, G., Vicente, M.F., Basilio, A., Rivas, C. L., Requena, T., Rodriguez, J. M., & Bartolome, B. (2010).Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. Research in Microbiology, 161, 372-382.

Cummings, I. H. (1995). Short chain fatty acids. In Human Colonic Bacteria: Role in Nutrition, Physiology and Pathology. pp. 101-130, GR Gibson and GT Macfarlane, Eds. Boca Raton, FL: CRC Press.

Daglia, M., Stauder, M., Papetti, A., Signoretto, C., Giusto, G., Canepari, P, Pruzzo C. & Gazzani, G. (2010). Isolation of red wine components with anti-adhesion and anti-biofilm activity against *Streptococcus mutans*. Food Chemistry, 119, 1182-1188.

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M. A., Biddinger, S.B., Dutton, R. J. & Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature, 505, 559-563.

Davila, A. M., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P. H., Sanz, Y., & Tomé, D. (2013). Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. Pharmacological Research, 68, 95-107.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., Collini, S., Pieraccini, G., & Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences of the United States of America, 107, 14691-14696.

De Ryck, T., Grootaert, C., Jaspaert, L., Kerckhof, F. M., Van Gele, M., De Schrijver, J., Van den Abbeele, P., Simon Swift, S., Bracke, M.,

Van de Wiele, T. & Vanhoecke, B. (2014). Development of an oral mucosa model to study host-microbiome interactions during wound healing. Applied Microbiology and Biotechnology, 98, 6831-6846.

De Weirdt, R., Possemiers, S., Vermeulen, G., Moerdijk-Poortvliet, T. C., Boschker, H. T., Verstraete, W., & Van de Wiele, T. (2010). Human faecal microbiota display variable patterns of glycerol metabolism. FEMS Microbiology Ecology, 74, 601-611.

Derrien, M., van Passel, M. W., van de Bovenkamp, J. H., Schipper, R., de Vos, W., & Dekker, J. (2010). Mucin-bacterial interactions in the human oral cavity and digestive tract. Gut Microbes, 1, 254-268.

Derrien, M., Vaughan, E.E., Plugge, C.M. & de Vos, W.M. (2004). *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. International Journal of Systematic and Evolutionary Microbiology, 54, 1469-1476.

Deshpande, G., Rao, S., Patole, S., & Bulsara, M. (2010). Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. Pediatrics, 125, 921-930.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., Lakshmanan, A. & Wade, W. G. (2010). The human oral microbiome. Journal of Bacteriology, 192, 5002-5017.

Diaz, P. I. (2011). Microbial diversity and interactions in subgingival biofilm communities. Frontiers of Oral Biology, 15, 17-40.

Diplock A.T., Aggett P.J., Ashwell M., Bornet F., Fern E.B. & Roberfroid M.B. (1999). Scientific concepts of functional foods in Europe: consensus document. British Journal of Nutrition, 81, S1–S27.

Dogi C.A., Maldonado Galdeano C. & Perdigón G. (2008). Gut immune stimulation by non pathogenic Gram(+) and Gram(-) bacteria. Comparison with a probiotic strain. Cytokine, 41, 223-231.

Doré, J., Simrén, M., Buttle, L., & Guarner, F. (2013). Hot topics in gut microbiota. United European Gastroenterology Journal, 1, 311-318.

Duda-Chodak, A. (2012). The inhibitory effect of polyphenols on human gut microbiota. Journal of Physiology and Pharmacology, 63, 497−503.

Duncan, S. H., Holtrop, G., Lobley, G. E., Calder, A. G., Stewart, C. S., & Flint, H. J. (2004). Contribution of acetate to butyrate formation by human faecal bacteria. British Journal of Nutrition, 91, 915-923.

Duncan, S. H., Louis, P., Thomson, J. M., & Flint, H. J. (2009). The role of pH in determining the species composition of the human colonic microbiota. Environmental Microbiology, 11, 2112-2122.

Eckburg,P.B., Bik,E.M., Bernstein,C.N., Purdom,E., Dethlefsen,L., Sargent,M., Gill,S.R., Nelson,K.E., & Relman,D.A. (2005). Diversity of the human intestinal microbial flora. Science, 308,1635-1638.

El Aidy, S., Van den Abbeele, P., Van de Wiele, T., Louis, P., & Kleerebezem, M. (2013). Intestinal colonization: How key microbial players become established in this dynamic process. Bioessays, 35, 913-923.

Espley R. V., Butts C. A., Laing W. A., Martell S., Smith H., McGhie T. K. & Hellens R. P. (2014). Dietary flavonoids from modified apple reduce inflammation markers and modulate gut microbiota in mice. The Journal of Nutrition. 144, 146-154.

Etxeberria, U., Fernández-Quintela, A., Milagro, F. I., Aguirre, L., Martínez, J. A., & Portillo, M. P. (2013). Impact of polyphenols and

polyphenol-rich dietary sources on gut microbiota composition. Journal of Agricultural and Food Chemistry, 61, 9517-9533.

Fábián, T. K., Hermann, P., Beck, A., Fejérdy, P., & Fábián, G. (2012). Salivary defense proteins: their network and role in innate and acquired oral immunity. International Journal of Molecular Sciences, 13, 4295-4320.

Faith, J. J., Guruge, J. L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A. L., Clemente, J. C., Knight, R., Heath A. C., Leibel, R. L., Rosenbaum, M. & Gordon, J. I. (2013). The long-term stability of the human gut microbiota. Science, 341, 1237439.

Fallani, M., Young, D., Scott, J., Norin, E., Amarri, S., Adam, R., Aguilera, M., Khanna, S., Gil, A., Edwards, C. A, Doré, J. & INFABIO team. (2010). Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. Journal of Pediatric Gastroenterology and Nutrition, 51, 77-84.

FAO/WHO (2002) Guidelines for the evaluation of probiotics in food. ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.

Filoche S. K., Soma K., & Sissons C. H. (2007). Caries-related plaque microcosm biofilms developed in microplates. Oral Microbiology and Immunology, 22, 73-79.

Filoche, S. K., Soma, D., van Bekkum, M., & Sissons, C. H. (2008). Plaques from different individuals yield different microbiota responses to oral-antiseptic treatment. FEMS Immunology and Medical Microbiology, 54, 27-36.

Fitzgerald, R. J., & Keyes, P. H. (1960). Demonstration of the etiologic role of streptococci in experimental caries in the hamster. The Journal of the American Dental Association, 61, 9-19.

Flint, H. J., Duncan, S. H., Scott, K. P., & Louis, P. (2015). Links between diet, gut microbiota composition and gut metabolism. Proceedings of the Nutrition Society, 74, 13-22.

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. Gut Microbes, 3, 289.

Fontecha J., Pelaéz C., Juárez M.; Requena T., Gómez C., & Ramos M. (1990). Biochemical and microbiological characteristics of artisanal hard goat's cheese. Journal of Dairy Science, 73, 1150-1157.

Forester, S. C., & Waterhouse, A. L. (2009). Metabolites are key to understanding health effects of wine polyphenolics. Journal of Nutrition, 139, 1824S-1831S.

Foster, J., & Neufeld, K. A. (2013). Gut-brain axis: How the microbiome influences anxiety and depression. Trends in Neurosciences, 36, 305-312.

Fraher M. H., O'Toole P. W. & Quigley E. M. M. (2012) Techniques used to characterize the gut microbiota: a guide for the clinician. Nature Reviews Gastroenterology and Hepatology, 9, 312-322.

Frank, D. N., Amand, A. L. S., Feldman, R. A., Boedeker, E. C., Harpaz, N., & Pace, N. R. (2007). Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences of the United States of America, 104,13780-13785.

Franke, A. A., Custer, L. J., Cooney, R. V., Tanaka, Y., Xu, M., & Dashwood, R.H. (2002). Inhibition of colonic aberrant crypt formation by the dietary flavonoids (+)–catechin and hesperidin. Advances in Experimental Medicine and Biology, 505, 123–133.

Fritz, J. V., Desai, M. S., Shah, P., Schneider, J.G., & Wilmes, P. (2013). From meta-omics to causality: experimental models for human microbiome research. Microbiome, 1, 14.

Fujimori, S., Gudis, K., Mitsui, K., Seo, T., Yonezawa, M., Tanaka, S., Tatsuguchi, A., & Sakamoto, C. (2009). A randomized controlled trial on the efficacy of synbiotic versus probiotic or prebiotic treatment to improve the quality of life in patients with ulcerative colitis. Nutrition, 25, 520-525.

Furiga, A., Lonvaud, A., & Badet, C. (2009). *In vitro* study of antioxidant capacity and antibacterial activity on oral anaerobes of a grape seed extract. Food Chemistry, 113, 1037-1040.

Gao, Z., Yin, J., Zhang, J., Ward, R. E., Martin, R. J., Lefevre M., Cefalu W.T., and Ye, J. (2009). Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes, 58, 1509-1517.

García-Cayuela, T., Korany, A. M., Bustos I., P Gómez de Cadiñanos, L., Requena, T., Peláez, C., & Martínez-Cuesta, M. C. (2014). Adhesion abilities of dairy *Lactobacillus plantarum* strains showing an aggregation phenotype. Food Research International, 57, 44-50.

Gibson, G.R., Cummings, J.H., & Macfarlane, G.T. (1988). Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Applied and Environmental Microbiology, 54, 2750-2755.

Gibson, G.R. & Roberfroid, M.B. (1995). Dietary modulation of the colonic microbiota: introducing the concept of prebiotics. Journal of Nutrition 125, 1401-1412.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A. & Roberfroid, M. B. (2004). Dietary modulation of the human colonic

microbiota: updating the concept of prebiotics. Nutrition Research Reviews, 17, 259-275.

Gibson, G. R., & Roberfroid, M. (Eds.). (2008). Handbook of prebiotics. CRC Press.

Goodman, A. L., Kallstrom, G., Faith, J. J., Reyes, A., Moore, A., Dantas, G., & Gordon, J. I. (2011). Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proceedings of the National Academy of Sciences of the United States of America, 108, 6252-6257.

Goossens, D. A. M., Jonkers, D. M. A. E., Russel, M. G. V. M., Stobberingh, E. E., & Stockbrügger, R. W. (2006). The effect of a probiotic drink with *Lactobacillus plantarum* 299v on the bacterial composition in faeces and mucosal biopsies of rectum and ascending colon. Alimentary Pharmacology and Therapeutics, 23, 255-263.

Grice, E. A., & Segre, J. A. (2011). The skin microbiome. Nature Reviews Microbiology, 9, 244-253.

Grimoud, J., Durand, H., Courtin, C., Monsan, P., Ouarné, F., Theodorou V., & Roques, C. (2010). *In vitro* screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics. Anaerobe, 16, 493-500.

Gross, G., Jacobs, D. M., Peters, S., Possemiers, S., van Duynhoven, J., Vaughan, E. E., & Van de Wiele, T. (2010). *In vitro* bioconversion of polyphenols from black tea and red wine/grape juice by human intestinal microbiota displays strong interindividual variability. Journal of Agricultural and Food Chemistry, 58, 10236-10246.

Grün C. H., van Dorsten F. A., Jacobs D. M., Le Belleguic M., van Velzen E. J. J., Bingham M. O., Janssena, H-G. & van Duynhoven J. P. M. (2008). GC-MS methods for metabolic profiling of microbial

fermentation products of dietary polyphenols in human and *in vitro* intervention studies. Journal of Chromatography B, 871, 212-219.

Guarner, F. & Malagelada, J. R. (2003). Gut flora in health and disease. Lancet, 361, 512-519.

Hamer, H. M., Jonkers, D. M. A. E., Venema, K., Vanhoutvin, S. A. L. W., Troost, F. J., & BRUMMER, R. J. (2008). Review article: the role of butyrate on colonic function. Alimentary Pharmacology and Therapeutics, 27, 104-119.

Hanski, I., von Hertzen, L., Fyhrquist, N., Koskinen, K., Torppa, K., Laatikainen, T., Piia Karisolac, P. Auvinend, P., Paulind, L., Mäkeläb, M. J., Vartiainene, E., Kosunenf, T.U., Aleniusc, H. & Haahtela, T. (2012). Environmental biodiversity, human microbiota, and allergy are interrelated. Proceedings of the National Academy of Sciences of the United States of America, 109, 8334-8339.

Hardy, H., Harris, J., Lyon, E., Beal, J., & Foey, A. D. (2013). Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. Nutrients, 5, 1869-1912.

Hatanaka, M., Nakamura, Y., Maathuis, A. J. H., Venema, K., Murota, I., & Yamamoto, N. (2012). Influence of *Bacillus subtilis* C-3102 on microbiota in a dynamic *in vitro* model of the gastrointestinal tract simulating human conditions. Beneficial Microbes, 3, 229-236.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C. & Sanders, M. E. (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology, 11, 506-514.

Ho, K. Y., Tsai, C. C., Huang, J. S., Chen, C. P., Lin, T. C., & Lin, C. C. (2001). Antimicrobial activity of tannin components from Vaccinium vitis-idaea L. Journal of Pharmacy and Pharmacology, 53, 187-191.

Holgerson, P. L., Harnevik, L., Hernell, O., Tanner, A. C. R., & Johansson, I. (2011). Mode of birth delivery affects oral microbiota in infants. Journal of Dental Research, 90, 1183-1188.

Hooper, L. V., & Macpherson, A. J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. Nature Reviews Immunology, 10, 159-169.

Hooper, L. V., Littman, D. R., & Macpherson, A. J. (2012). Interactions between the microbiota and the immune system. Science, 336, 1268-1273.

Hosseini, E., Grootaert, C., Verstraete, W., & Van de Wiele, T. (2011). Propionate as a health-promoting microbial metabolite in the human gut. Nutrition Reviews, 69, 245-258.

Huang, Y., Wang, X., Wang, J., Wu, F., Sui, Y., Yang, L., & Wang, Z. (2013). *Lactobacillus plantarum* strains as potential probiotic cultures with cholesterol-lowering activity. Journal of Dairy Science, 96, 2746-2753.

Hunt, K. M., Foster, J. A., Forney, L. J., Schütte, U. M., Beck, D. L., Abdo, Z., Fox, L. K., Williams, J. E., McGuire, M. K & McGuire, M. A. (2011). Characterization of the diversity and temporal stability of bacterial communities in human milk. PLoS One, 6, e21313.

Huttenhower et al. The Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. Nature, 486, 207-214.

Huycke, M. M. & Gaskins, H. R. (2004) Commensal bacteria, redox stress, and colorectal cancer: mechanisms and models. Experimental Biology and Medicine, 229, 586-597.

Ishikawa, H., Kutsukake, E., Fukui, T., Sato, I., Shirai, T., Kurihara, T., Okada, N., Danbara, H., Toba, M., Kohda, N., Maeda, Y., & Matsumoto, T. (2010). Oral administration of heat-killed *Lactobacillus plantarum* strain b240 protected mice against *Salmonella enterica* serovar typhimurium. Bioscience, Biotechnology, and Biochemistry, 74, 1338-1342.

Jacobs, D. M., Deltimple, N., van Velzen, E., van Dorsten, F. A., Bingham, M., Vaughan, E. E., & van Duynhoven, J. (2008). 1H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. NMR in Biomedicine, 21, 615-626.

Jarchum, I., & Pamer, E. G. (2011). Regulation of innate and adaptive immunity by the commensal microbiota. Current Opinion in Immunology, 23, 353-360.

Jeffery, I. B., Claesson, M. J., O'Toole, P. W., & Shanahan, F. (2012). Categorization of the gut microbiota: enterotypes or gradients?. Nature Reviews Microbiology, 10, 591-592.

Jenkinson, H. F. &Lamont, R. J. (2005). Oral microbial communities in sickness and in health. Trends in Microbiology, 13, 589-595.

Jeurink, P. V., Van Bergenhenegouwen, J., Jimenez, E., Knippels, L. M. J., Fernandez, L., Garssen, J., Knol, J., Rodríguez, J. M. & Martin, R. (2013). Human milk: a source of more life than we imagine. Beneficial Microbes, 4, 17-30.

Jimenez, E., Marín, M.L, Martín, R., Odriozola, J.M., Olivares, M., Xaus, J., Fernandez, L., & Rodriguez, J.M. (2008). Is meconium from

healthy newborns actually sterile?. Research in Microbiology, 159, 187-193.

Johnston, B. C., Goldenberg, J. Z., Vandvik, P. O., Sun, X., & Guyatt, G. H. (2011). Probiotics for the prevention of pediatric antibiotic-associated diarrhea. The Cochrane Database of Systematic Reviews, 11, CD004827.

Kale-Pradhan, P. B., Jassaly, H. K., & Wilhelm, S. M. (2010). Role of *Lactobacillus* in the Prevention of Antibiotic-Associated Diarrhea: A Meta-analysis. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 30, 119-126.

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., & Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. Nature, 474, 327-336.

Keijser, B. J. F., Zaura, E., Huse, S. M., Van Der Vossen, J. M. B. M., Schuren, F. H. J., Montijn, R., Ten Cate, J.M. & Crielaard, W. (2008). Pyrosequencing analysis of the oral microflora of healthy adults. Journal of Dental Research, 87, 1016-1020.

Kemperman R. A., Gross G., Mondot S., Possemiers S., Marzorati M., Van de Wiele T., Doré J. & Vaughan E. E. (2013). Impact of polyphenols from black tea and red wine/grape juice on a gut model microbiome. Food Research International, 53, 659-669.

Knights, D., Ward, T. L., McKinlay, C. E., Miller, H., Gonzalez, A., McDonald, D., & Knight, R. (2014). Rethinking “Enterotypes”. Cell Host & Microbe, 16, 433-437.

Kong F., & Singh R.P. (2010). A human gastric simulator (HGS) to study food digestion in human stomach. Journal of Food Science, 75, E627-E635.

Kongstad J., Hvidtfeldt U. A., Gronbaek M., Jontell M., Stoltze K. & Holmstrup P. (2008). Amount and type of alcohol and periodontitis in the Copenhagen City heart study. Journal of Clinical Periodontology, 35, 1032-1039.

Koren O., Goodrich J.K., Cullender T.C., Spor A., Laitinen K., Kling Bäckhed H., Gonzalez A., Werner J.J., Angenent L.T., Knight R., Bäckhed, F., Isolauri, E., Salminen, S. Ley, R. E. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell, 150, 470–480.

Kozyrskyj, A. L., Bahreinian, S. & Azad, M. B. (2011). Early life exposures: impact on asthma and allergic disease. Current Opinion in Allergy and Clinical Immunology, 11, 400-406.

Kumar, H., Salminen, S., Verhagen, H., Rowland, I., Heimbach, J., Bañares, S, Young, T., Nomoto, K.& Lalonde, M. (2015). Novel probiotics and prebiotics: road to the market. Current Opinion in Biotechnology, 32, 99-103.

Kuramitsu, H. K., He, X., Lux, R., Anderson, M. H., & Shi, W. (2007). Interspecies interactions within oral microbial communities. Microbiology and Molecular Biology Reviews, 71, 653-670.

Lakshminarayanan, B., Guinane, C. M., O'Connor, P. M., Coakley, M., Hill, C., Stanton, C. O'Toole, P.W., & Ross, R. P. (2013). Isolation and characterization of bacteriocin-producing bacteria from the intestinal microbiota of elderly Irish subjects. Journal of Applied Microbiology, 114, 886-898.

Lamont, R. F., Sobel, J. D., Akins, R. A., Hassan, S. S., Chaiworapongsa, T., Kusanovic, J. P., & Romero, R. (2011). The vaginal microbiome: new information about genital tract flora using molecular

based techniques. BJOG: An International Journal of Obstetrics & Gynaecology, 118, 533-549.

Lazarevic, V., Whiteson, K., Hernandez, D., François, P., & Schrenzel, J. (2010). Study of inter-and intra-individual variations in the salivary microbiota. BMC Genomics, 11, 523.

Lazarevic, V., Whiteson, K., Huse, S., Hernandez, D., Farinelli, L., Østerås, M, Schrenzela, J., & François, P. (2009). Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. Journal of Microbiological Methods, 79, 266-271.

Lebeer, S., Vanderleyden, J., & De Keersmaecker, S.C. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nature Reviews Microbiology, 8, 171–84.

LeBlanc, J. G., Milani, C., de Giori, G. S., Sesma, F., van Sinderen, D., & Ventura, M. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Current Opinion in Biotechnology, 24, 160-168.

Lee, H. C., Jenner, A. M., Low, C. S., & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. Research in Microbiology, 157, 876-884.

Leitch, E.C., Walker, A.W., Duncan, S.H., Holtrop, G. & Flint, H.J. (2007). Selective colonization of insoluble substrates by human faecal bacteria. Environmental Microbiology, 9, 667-679.

Lepage, P., Leclerc, M. C., Joossens, M., Mondot, S., Blottière, H. M., Raes, J., Dusko, E. & Doré, J. (2013). A metagenomic insight into our gut's microbiome. Gut, 62, 146-158.

Lesmes, U., Beards E. J., Gibson, G.R., Tuohy K. M. & Shimoni, E. (2008). Effects of resistant starch type III polymorphs on human colon

microbiota and short chain fatty acids in human gut models. Journal of Agricultural and Food Chemistry, 56, 5415–5421.

Lesuffleur, T., Kornowski, A., Luccioni, C., Muleris, M., Barbat, A., Beaumatin, J, Dussaulx, E., Dutrillaux, B. & Zweibaum, A. (1991). Adaptation to 5-fluorouracil of the heterogeneous human colon tumor cell line ht-29 results in the selection of cells committed to differentiation. International Journal of Cancer, 49, 721-730.

Liong, M.T. (2008). Roles of probiotics and prebiotics in colon cancer prevention: postulated mechanisms and in-vivo evidence. International Journal of Molecular Sciences, 9, 854-863.

Louis, P., & Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiology Letters, 294, 1-8.

Lourenço, C. F., Gago, B., Barbosa, R. M., de Freitas, V., & Laranjinha, J. (2008). LDL isolated from plasma-loaded red wine procyanidins resist lipid oxidation and tocopherol depletion. Journal of Agricultural and Food Chemistry, 56, 3798-3804.

Louvard, D., Kedinger, M., & Hauri, H. P. (1992). The differentiating intestinal epithelial cell: establishment and maintenance of functions through interactions between cellular structures. Annual Review of Cell Biology, 8, 157-195.

Lozupone C.A., Stombaugh J.I., Gordon J.I., Jansson J.K. & Knight R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature, 489, 220–230.

Macfarlane G., Macfarlane S. & Gibson G.R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human gut. Microbial Ecology, 35,180–187.

Macfarlane, G. T., Gibson, G. R., & Cummings, J. H. (1992). Comparison of fermentation reactions in different regions of the human colon. Journal of Applied Bacteriology, 72, 57-64.

Mager, D. L., Haffajee, A. D., Devlin, P. M., Norris, C. M., Posner, M. R., & Goodson, J. M. (2005). The salivary microbiota as a diagnostic indicator of oral cancer: a descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. Journal of Translational Medicine, 3, 27.

Maloy, K. J., & Powrie, F. (2011). Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature, 474, 298-306.

Marchesi, J. R. (2011). Human distal gut microbiome. Environmental Microbiology, 13, 3088-3102.

Marsh P.D. (2006). Dental plaque as a biofilm and a microbial community implications for health and disease. BMC Oral Health, 6, 14.

Marsh, P. D. & Percival, R. S. (2006). The oral microflora- friend or foe? Can we decide? International Dental Journal, 56, 233-239.

Marsh, P. D., & Devine, D. A. (2011). How is the development of dental biofilms influenced by the host?. Journal of Clinical Periodontology, 38, 28-35.

Martinez, R. C., Cardarelli, H. R., Borst, W., Albrecht, S., Schols, H., Gutiérrez, O. P., Maathuis, A. J. H., de Melo Franco, B. D. G., De Martinis, E. C. P., Zoetendal, E. G., Venema, K., Saad, S. M. I. & Smidt, H. (2013). Effect of galactooligosaccharides and *Bifidobacterium animalis*Bb-12 on growth of *Lactobacillus amylovorus* DSM 16698, microbial community structure, and metabolite production in an *in vitro* colonic model set up with human or pig microbiota. FEMS Microbiology Ecology, 84, 110-123.

Marx, S P, Winkler, S. & Hartmeier, W. (2000). Metabolization of β-(2,6)- lined fructoseoligosaccharides by different bifidobacteria FEMS Microbiology Letters, 182, 163-169.

Marzorati, M., Vanhoecke, B., De Ryck, T., Sadabad, M. S., Pinheiro, I., Possemiers S., Van den Abbeele, P., Derycke, L., Bracke, M., Pieters J., Hennebel, T., Harmsen, H. J., Verstraete, W. & Van de Wiele, T.(2014). The HMI™ module: a new tool to study the Host-Microbiota Interaction in the human gastrointestinal tract *in vitro*. BMC Microbiology, 14, 133.

Mazmanian, S. K., & Lee, Y. K. (2014). Interplay between Intestinal Microbiota and Host Immune System. Journal of Bacteriology and Virology, 44, 1-9.

McDonald, J. A., Fuentes, S., Schroeter, K., Heikamp-deJong, I., Khursigara, C. M., de Vos, W. M., & Allen-Vercoe, E. (2014). Simulating distal gut mucosal and luminal communities using packed-column biofilm reactors and an *in vitro* chemostat model. Journal of Microbiological Methods, 108, 36-44.

Metchnikoff, E. & Mitchell, P. C. (1907). The prolongation of life; optimistic studies. W. Heinemann,; G. P. Putnam's Sons, New York and London.

Methé et al.The Human Microbiome Project Consortium. (2012). A framework for human microbiome research. Nature, 486, 215-221.

Minekus, M., Marteau, P., Havenaar, R. & Huis in't Veld, J.H.J. (1995). A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. Alternatives to laboratory animals: ATLA, 23, 197-209.

Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonnin, S., Havenaar, R., Marteau, P., Alric, M., Fonty, G. & Huis in't Veld J. H.

(1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Applied Microbiology and Biotechnology, 53, 108-14.

Moles, L., Gomez, M., Heilig, H., Bustos, G., Fuentes, S., de Vos, W., Fernández L., Rodríguez, J. M. & Jiménez, E. (2013). Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. PLoS One, 8, e66986.

Molly K., Van de Woestyne M., & Verstraete W. (1993). Development of a 5-step multichamber reactor as a simulation of the human intestinal microbial ecosystem. Applied Microbiology and Biotechnology, 39, 254-258.

Mueller, S., Saunier, K., Hanisch, C., Norin, E., Alm, L., Midtvedt, T., Cresci A., Silvi, S., Orpianesi, C., Verdenelli, M. C.,Clavel, T., Kobnick, C., Zunft, H-J. F., Doré, J. & Michael Blaut, m. (2006). Differences in fecal microbiota in different European study populations in relation to age, gender, and country: A cross-sectional study. Applied and Environmental Microbiology, 72, 1027-1033.

Navarrete L. A. S., & Rizzardinni G. D. C. M. (2011). U.S. Patent Application 13/820,132.

Ndiaye, M., Chataigneau, T., Andriantsitohaina, R., Stoclet, J. C., & Schini-Kerth, V. B. (2003). Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism. Biochemical and Biophysical Research Communications, 310, 371-377.

Neish, A.S. (2009). Microbes in gastrointestinal health and disease. Gastroenterology, 136, 65-80.

Neufeld, K.M., Kang, N., Bienenstock, J., & Foster, J.A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterology and Motility, 23, 255-64

Newton, D. F., Macfarlane, S. & Macfarlane, G. T. (2013). Effects of antibiotics on bacterial species composition and metabolic activities in chemostats containing defined populations of human gut microorganisms. Antimicrobial Agents and Chemotherapy, 57, 2016-2025.

Okada, H., Kuhn, C., Feillet, H., & Bach, J. F. (2010). The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Clinical & Experimental Immunology, 160, 1-9.

Ottman, N., Smidt, H., De Vos, W. M., & Belzer, C. (2012). The function of our microbiota: who is out there and what do they do?. Frontiers in Cellular and Infection Microbiology, 2,104.

Ouwehand A., Nermes M., Collado M.C., Rautonen N., Salminen S. & Isolauri, E. (2009) Specific probiotics alleviate allergic rhinitis during the birch pollen season. World Journal of Gastroenterology, 15, 3261-8.

Paineau, D., Payen, F., Panserieu, S., Coulombier, G., Sobaszek, A., Lartigau, I., Brabet, M., GalmicheJ-P., Tripodi, D., Sacher-Huvelin, S., Chapalain, V., Zourabichvili, O., Respondek, F., Wagner, A. & Bornet F. R.J. (2008). The effects of regular consumption of short-chain fructo-oligosaccharides on digestive comfort of subjects with minor functional bowel disorders. British Journal of Nutrition, 99, 311.

Panda, S., Guarner, F., & Manichanh, C. (2014). Structure and functions of the gut microbiome. Endocrine, Metabolic and Immune Disorders-Drug Targets, 14, 290-299.

Pandeya, D. R., D'Souza, R., Rahman, M. M., Akhter, S., Kim, H. J., & Hong, S. T. (2012). Host-microbial interaction in the mammalian intestine and their metabolic role inside. Biomedical Research, 23, 9-21.

Park, M. K., Vu, N. G. O., Kwon, Y. M., Lee, Y. T., Yoo, S., Cho, Y. H, Hong S. M., Hwang H. S., Ko E. J., Jung Y. J., Moon D. W., Jeong E. J., Kim M. C., Lee Y. N., Jang J. H., Oh J. S., Kim C. H. & Kang, S. M. (2013). *Lactobacillus plantarum* DK119 as a probiotic confers protection against influenza virus by modulating innate immunity. PloS One, 8, e75368.

Payne A. N., Zihler A., Chassard C., & Lacroix, C. (2012). Advances and perspectives in *in vitro* human gut fermentation modeling. Trends in Biotechnology, 30, 17-25.

Pompei, A., Cordisco, L., Raimondi, S., Amaretti, A., Pagnoni, U. M., Matteuzzi, D., & Rossi, M. (2008). *In vitro* comparison of the prebiotic effects of two inulin-type fructans. Anaerobe, 14, 280-286.

Possemiers, S., Bolca, S., Eeckhaut, E., Depypere, H., & Verstraete, W.(2007). Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community FEMS Microbiology Ecology, 61,372–383.

Pozo-Bayón, M. Á., Monagas, M., Bartolomé, B., & Moreno-Arribas,M. V. (2012). Wine features related to safety and consumer health: an integrated perspective. Critical Reviews in Food Science and Nutrition, 52, 31-54.

Preidis, G. A., & Versalovic, J. (2009). Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. Gastroenterology, 136, 2015-2031.

Qin et al. MetaHIT Consortium. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature, 464, 59-65.

Queipo-Ortuno, M. I., Boto-Ordonez, M., Murri, M., Gomez-Zumaquero, J. M., Clemente-Postigo, M., Estruch, R., Cardona Diaz, F., Andres-Lacueva, C. & Tinahones, F. J. (2012). Influence of red wine

polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. The American Journal of Clinical Nutrition, 95, 1323-1334.

Rajilić-Stojanović, M., Smidt, H., & De Vos, W. M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. Environmental Microbiology, 9, 2125-2136.

Rajilić-Stojanović, M., Maathuis, A., Heilig, H. G., Venema, K., de Vos, W. M., & Smidt, H. (2010). Evaluating the microbial diversity of an *in vitro* model of the human large intestine by phylogenetic microarray analysis. Microbiology, 156, 3270-3281.

Rajilić-Stojanović, M., Heilig, H. G., Tims, S., Zoetendal, E. G., & Vos, W. M. (2013). Long-term monitoring of the human intestinal microbiota composition. Environmental Microbiology, 15, 1146-1159.

Ramirez-Farias, C., Slezak, K., Fuller, Z., Duncan, A., Holtrop, G., & Louis, P. (2009). Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. British Journal of Nutrition, 101, 541-550.

Reid, G., Dols, J., & Miller, W. (2009). Targeting the vaginal microbiota with probiotics as a means to counteract infections. Current Opinion in Clinical Nutrition and Metabolic Care, 12, 583-587.

Reid, G., Younes, J. A., Van der Mei, H. C., Gloor, G. B., Knight, R., & Busscher, H. J. (2011). Microbiota restoration: natural and supplemented recovery of human microbial communities. Nature Reviews Microbiology, 9, 27-38.

Reyniers J.A. (1959). The pure culture concept and gnotobiotics. Annals of the New York Academy of Sciences, 78, 3-16.

Ridaura V.K., Faith J. J., Rey F. E., Cheng J., Duncan A. E., Kau A. L. & Gordon J. I. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science, 341, 1241214.

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I, Wolvers, D., Watzl, B., Szajewska, H., Stahl B., Guarner F., Respondek,F., Whelan, K., Coxam, V., Davicco, M. J, Léotoing L., Wittrant Y., Delzenne, N. M., Cani, P. D., Neyrinck, A. M. & Meheust, A. (2010). Prebiotic effects: metabolic and health benefits. British Journal of Nutrition, 104, S1-S63.

Rogers Y. H. & Venter J. C. (2005). Genomics: massively parallel sequencing. Nature 437, 326-327.

Rokka, S., Pihlanto, A., Korhonen, H., & Joutsjoki, V. (2006). *In vitro* growth inhibition of helicobacter pylori by lactobacilli belonging to the *Lactobacillus plantarum* group. Letters in Applied Microbiology, 43, 508-513.

Romano-Keeler, J., Wynn, J.L., & Maron, J.L. (2014). Great expectorations: the potential of salivary 'omic' approaches in neonatal intensive care. Journal of Perinatology, 34, 169-173.

Rook, G. A. (2012). Hygiene hypothesis and autoimmune diseases. Clinical Reviews in Allergy & Immunology, 42, 5-15.

Rook, G. A., Raison, C. L., & Lowry, C. A. (2013). Childhood microbial experience, immunoregulation, inflammation and adult susceptibility to psychosocial stressors and depression in rich and poor countries. Evolution, Medicine, and Public Health, 2013, 14-17.

Round, J.L., & Mazmanian, S.K: (2009).The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews Immunology, 9, 313-323.

Rowland, I. R. (2009). The role of the gastrointestinal microbiota in colorectal cancer. Current Pharmaceutical Design, 15, 1524-1527.

Russell, W. R., Gratz, S. W., Duncan, S. H., Holtrop, G., Ince, J., Scobbie, L., Duncan G., Johnstone, A.M., Lobley G.E., Wallace R.J., Duthie G.G. & Flint, H. J. (2011). High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. The American Journal of Clinical Nutrition, 93, 1062-1072.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., & Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biology and Toxicology, 21, 1-26.

Saulnier, D.M., Gibson, G.R., & Kolida, S. (2008).*In vitro* effects of selected synbiotics on the human faecal microbiota composition. FEMS Microbiology Ecology, 66,516-527.

Savard, P., Lamarche, B., Paradis, M. E., Thiboutot, H., Laurin, É., & Roy, D. (2011). Impact of *Bifidobacterium animalis* subsp *lactis* BB-12 and, *Lactobacillus acidophilus* LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. International Journal of Food Microbiology, 149, 50-57.

Scholtens, P. A., Alliet, P., Raes, M., Alles, M. S., Kroes, H., Boehm, G. Knippels L. M. J, Knol J. & Vandenplas, Y. (2008). Fecal secretory immunoglobulin A is increased in healthy infants who receive a formula with short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides. The Journal of Nutrition, 138, 1141-1147.

Schrezenmeir, J. & De Vrese, M. (2001). Probiotics, prebiotics, and synbiotics--approaching a definition. American Journal of Clinical Nutrition 73, 361-364.

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2013). The influence of diet on the gut microbiota. Pharmacological Research, 69, 52-60.

Scott, K. P., Martin, J. C., Duncan, S. H., & Flint, H. J. (2014). Prebiotic stimulation of human colonic butyrate-producing bacteria and bifidobacteria, *in vitro*. FEMS Microbiology Ecology, 87, 30-40.

Sekirov, I., Shannon, L. Russell, S.L., Antunes, L.C.M. & Finlay, B.B. (2010). Gut Microbiota in Health and Disease. Physiological Reviews, 90,859-904.

Selma, M. V., Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: role in human health. Journal of Agricultural and Food Chemistry, 57, 6485-6501.

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., & Rawls, J. F. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host & Microbe, 12, 277-288.

Shale, M., Schiering, C., & Powrie, F. (2013). CD4+ T-cell subsets in intestinal inflammation. Immunological Reviews, 252, 164-182.

Signoretto, C., Bianchi, F., Burlacchini, G., Sivieri, F., Spratt, D., & Canepari, P. (2010). Drinking habits are associated with changes in the dental plaque microbial community. Journal of Clinical Microbiology, 48, 347-356.

Siqueira, J. F., & Rôças, I. N. (2009). Diversity of endodontic microbiota revisited. Journal of Dental Research, 88, 969-981.

Sleeth, M. L., Thompson, E. L., Ford, H. E., Zac-Varghese, S. E., & Frost, G. (2010). Free fatty acid receptor 2 and nutrient sensing: a

proposed role for fibre, fermentable carbohydrates and short-chain fatty acids in appetite regulation. Nutrition Research Reviews, 23, 135-145.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermúdez-Humarán, L. G., Gratadoux, J. J Sébastien Blugeon S., Bridonneau C., Furet J-P., Corthier G., Grangette C., Vasquez N., Pochart P., Trugnan G., Thomas G., Blottière H.M, Doré J., Marteaull P., Seksik P., & Langella, P. (2008). *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proceedings of the National Academy of Sciences of the United States of America, 105, 16731-16736.

Stahringer, S. S., Clemente, J. C., Corley, R. P., Hewitt, J., Knights, D., Walters, W. A., Knight, R. & Krauter, K. S. (2012). Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Research, 22, 2146-2152.

Stout, M. J., Conlon, B., Landeau, M., Lee, I., Bower, C., Zhao, Q., Kimberly A. Roehl, K. A., Nelson, D. M., Macones G. A. & Mysorekar, I. U. (2013). Identification of intracellular bacteria in the basal plate of the human placenta in term and preterm gestations.American Journal of Obstetrics and Gynecology, 208, 226-e1-7.

Strobel, S. &Mowat, A.M. (2006). Oral tolerance and allergic responses to food proteins. Current Opinion in Allergy and Clinical Immunology, 6, 207-213.

Tabasco, R., Sanchez-Patan, F., Monagas, M., Bartolome, B., Moreno-Arribas, M. V., Pelaez, C., & Requena, T. (2011). Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: resistance and metabolism. Food Microbiology, 28, 1345-1352.

Tap, J., Mondot, S., Levenez, F., Pelletier, E., Caron, C., Furet, J.P., Ugarte, E., Muñoz-Tamayo, R., Paslier, D.L., Nalin, R., Dore, J. &Leclerc, M. (2009).Towards the human intestinal microbiota phylogenetic core. Environmental Microbiology, 11, 2574-2584.

Terpend, K., Possemiers, S., Daguet, D., & Marzorati, M. (2013). Arabinogalactan and fructo-oligosaccharides have a different fermentation profile in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Environmental Microbiology Reports, 5, 595-603.

Thomas, L. V., Ockhuizen, T., & Suzuki, K. (2014). Exploring the influence of the gut microbiota and probiotics on health: a symposium report. British Journal of Nutrition, 112, 1-18.

Tissier, H. (1900). Recherches sur la flore intestinale des nourrissons (état normal et pathologique). Paris: G. Carre and C. Naud.

Tsurugizawa, T., Uematsu, A., Nakamura, E., Hasumura, M., Hirota, M., Kondoh, T., Uneyama, H., & Torii, K. (2009). Mechanisms of neural response to gastrointestinal nutritive stimuli: the gut-brain axis. Gastroenterology, 137, 262-273.

Tuohy, K. M., Conterno, L., Gasperotti, M., & Viola, R. (2012). Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. Journal of Agricultural and Food Chemistry, 60, 8776-8782.

Tzounis, X., Vulevic, J., Kuhnle, G. C., George, T., Leonczak, J., Gibson, G. R., Kwik-Uribea, C. & Spencer, J. P. E. (2008). Flavonolmonomer-induced changes to the human faecal microflora. British Journal of Nutrition, 99, 782-792.

Vaishnava, S., Behrendt, C. L., Ismail, A. S., Eckmann, L., & Hooper, L. V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface.

Proceedings of the National Academy of Sciences of the United States of America, 105, 20858-20863.

Vallès, Y., Artacho, A., Pascual-García, A., Ferrús, M. L., Gosalbes, M. J., Abellán, J. J., & Francino, M. P. (2014). Microbial Succession in the Gut: Directional Trends of Taxonomic and Functional Change in a Birth Cohort of Spanish Infants. PLoS Genetics, 10, e1004406.

Van den Abbeele, P., Grootaert, C., Marzorati, M., Possemiers, S., Verstraete, W., Gérard, P., Rabot, S., Bruneau, A., El Aidy, S., Derrien, M., Zoetendal, E., Kleerebezem, M., Smidt, H., & Van de Wiele, T. (2010). Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and *Clostridium* cluster IX. Applied and Environmental Microbiology, 76, 5237-5246.

Van den Abbeele, P., Roos, S., Eeckhaut, V., MacKenzie, D. A., Derde, M., Verstraete, W., Marzorati M., Possemiers S., Vanhoecke B., Immerseel, F. V. & Van de Wiele, T. (2012). Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. Microbial Biotechnology, 5, 106-115.

Van den Abbeele, P., Van de Wiele, T., Verstraete, W., & Possemiers, S. (2011). The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiology Reviews, 35, 681-704.

Van den Abbeele, P., Venema, K., Van de Wiele, T., Verstraete, W., & Possemiers, S. (2013). Different human gut models reveal the distinct fermentation patterns of arabinoxylan versus inulin. Journal of Agricultural and Food Chemistry, 61, 9819–9827.

Venema K. & Van den Abbeele P. (2013). Experimental models of the gut microbiome. Best Practice and Research Clinical Gastroenterology, 27, 115-126.

Ventura, M., Turroni,F., Canchaya,C., Vaughan,E.E., O'Toole,P.W., & van Sinderen,D. (2009). Microbial diversity in the human intestine and novel insights from metagenomics. Frontiers in Bioscience. 14, 3214-3863.

Vestman, N. R., Timby, N., Holgerson, P. L., Kressirer, C. A., Claesson, R., Domellöf, M., Öhman, C., Tanner, A. C. R., Hernell, O & Johansson, I. (2013). Characterization and *in vitro* properties of oral lactobacilli in breastfed infants. BMC Microbiology, 13, 1-12.

Vital, M., Howe, A. C., & Tiedje, J. M. (2014). Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta) genomic Data. mBio, 5, e00889-14.

Voreades, N., Kozil, A., & Weir, T. L. (2014). Diet and the development of the human intestinal microbiome. Frontiers in Microbiology, 5, 494.

Wade, W. G. (2013). The oral microbiome in health and disease. Pharmacological Research, 69, 137-143.

Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., Brown, D., Stares, D. M.,Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A. M., Lobley, G. E., Parkhill, J. & Flint, H. J. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. The ISME Journal, 5, 220-230.

Wallace, T.C., Guarner, F., Madsen, K., Cabana, M.D., Gibson, G., Hentges, E., & Sanders, M.E. (2011). Human gut microbiota and its relationship to health and disease. Nutrition Reviews, 69, 392-403.

Walle, T., Browing, A. M., Steed, L., Reed, S. G. & Walle, U. K. (2005). Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans. Journal of Nutrition, 135, 48-52.

Walsh, C. J., Guinane, C. M., O'Toole, P. W., & Cotter, P. D. (2014). Beneficial modulation of the gut microbiota. FEBS letters, 588, 4120-4130.

Walter, A., Cerdeno-Tarraga, A., &Bentley, S. (2006). Faecal matters. Nature Reviews Microbiology 4, 572-573.

Watanabe, S., Narisawa, Y., Arase, S., Okamatsu, H., Ikenaga, T., Tajiri, Y. & Kumemura, M. (2003). Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. Journal of Allergy and Clinical Immunology, 111, 587-591.

Watt, E., Berry, S. H., Farquharson, F., Glaire, M. A., Thomson, J. M., El-Omar, E., Louis, P. & Hold, G. L. (2013, May). Evaluation of Fecal Aspirate As a Proxy for Colonoscopy Biopsy Sampling to Assess Microbial Diversity. In Gastroenterology (Vol. 144, No. 5, pp. S832-S832), Philadelphia, PA 19103-2899 USA: WB Saunders Co-Elsevier Inc.

Wickham, M. & Faulks, R. (2012). Dynamic Gastric Model. U.S. Patent 8092222.

Wilson, M. (2005). Microbial Inhabitants of Humans: their Ecology and Role in Health and Disease. Cambridge University Press, Cambridge, UK.

Wu, C. D. (2009). Grape products and oral health. Journal of Nutrition, 139, 1818-1823.

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., Magris, M., Hidalgo, G., Baldassano, R. N., Anokhin, A. P, Heath, A. C, Warner, B., Reeder, J., Kuczynski, J.,

Caporaso, J. G., Lozupone, C. A., Lauber, C., Clemente, J. C., Knights, D., Knight, R. Jeffrey, I. & Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. Nature, 486, 222–227.

Zaura, E., Keijser, B.J., Huse, S.M. & Crielaard, W. (2009). Defining the healthy “core microbiome” of oral microbial communities. BMC Microbiology, 9, 259.

Zaura, E., Nicu, E. A., Krom, B. P., & Keijser, B. J. (2014). Acquiring and maintaining a normal oral microbiome: current perspective. Frontiers in Cellular and Infection Microbiology, 4, 85.

Zhou, Y., Gao, H., Mihindukulasuriya, K. A., La Rosa, P. S., Wylie, K. M., Vishnivetskaya, T., Podar, M., Warner, B., Phillip I Tarr, P. I., Nelson, D. E., Fortenberry, J.D., Holland, M. J., Burr S.E., Shannon, W.D., Sodergren, E. & Weinstock, G. M. (2013). Biogeography of the ecosystems of the healthy human body. Genome Biology, 14, R1.

II. OBJECTIVES/OBJETIVOS

OBJECTIVES AND WORKING PLAN

Diet plays an important role in the oral microbiota, being at the same time the most significant external factor affecting the intestinal microbiota. In both ecosystems, a correct balance between microbiota and host is crucial for maintenance of health status. Some food ingredients, such as polyphenols, probiotic bacteria and prebiotics, have been demonstrated interesting features for approaching microbial modulation studies. Due to the different antimicrobial activities exerted by polyphenols, based on their chemical structures and the bacterial species studied, it seems appropriate to evaluate the effect of polyphenols on the human microbiota. Besides, some bacteria are able to metabolize polyphenols, which could represent an additional advantage inside the complex intestinal ecosystem and could give rise to more bioactive metabolites with potential beneficial effects on human health. Concerning prebiotics, currently there is increasing interest in the search of new compounds able to reach the distal colon regions, where exert their intestinal microbiota modulatory effect.

In this context, the objectives of this PhD Thesis have been as follows:

1. To evaluate the impact of different dietary polyphenols on the human intestinal microbiota.

In order to achieve this objective, it has been performed batch incubations of colonic microbiota with different polyphenolic extracts, cranberry, grape seed (Vitaflavan®) and red wine (Provinols^{TM}) extracts. Chapters III and IV.

2. To study the modulatory effect on the human intestinal microbiota of *Lactobacillus plantarum* IFPL935 associated to its ability to metabolize polyphenols.

In order to achieve this objective, it has been performed incubations of the colonic microbiota with the extract of wine polyphenols (Provinols^{TM}) and *L. plantarum* IFPL935, in batch and using a dynamic gastrointestinal simulator. Chapters IV and V.

3. To analyze the impact of moderate red wine intake on the composition of the oral and intestinal microbiota through a human intervention study. Chapters VI and VII.

4. To evaluate the prebiotic effect of lactulose-derived oligosaccharides (OsLu) on the human microbiota differentiated by colonic regions.

In order to achieve this objective, it has been developed a computer-controlled dynamic *in vitro* gastrointestinal simulator, where it was evaluated the effect on the colonic microbiota of the dietary substitution of easily digestible carbohydrates by OsLu. Chapters VIII and IX.

OBJETIVOS Y PLAN DE TRABAJO

La dieta juega un papel significativo en la microbiota oral, siendo a la vez el factor externo más relevante que afecta a la microbiota intestinal. En ambos entornos, un correcto equilibrio entre microbiota y hospedador es crucial para el mantenimiento de la salud. Algunos ingredientes alimentarios, como los polifenoles, las bacterias probióticas y los prebióticos, poseen características que los hacen interesantes para abordar estudios de modulación de la microbiota oral e intestinal. Debido a la variabilidad antimicrobiana que presentan los polifenoles en función de las diferentes estructuras químicas y especies bacterianas estudiadas, resulta relevante evaluar su acción sobre la microbiota humana. Por otra parte, la capacidad de algunas bacterias para metabolizar polifenoles podría aportarles ventajas dentro del complejo ecosistema intestinal, además de favorecer la formación de metabolitos bioactivos beneficiosos para el organismo. En el caso de los prebióticos, se tiende en la actualidad a la búsqueda de nuevos compuestos que puedan alcanzar zonas distales del colon donde ejercer su acción moduladora sobre la microbiota intestinal.

En este contexto, los objetivos de esta Tesis Doctoral han sido los siguientes:

1. Evaluar el impacto de diferentes polifenoles de la dieta en la microbiota intestinal humana.

Para la consecución de este objetivo se han llevado a cabo incubaciones en condiciones estáticas de distintos extractos de polifenoles - arándano rojo, pepita de uva (Vitaflavan®) y vino tinto (Provinols^{TM})- con microbiota colónica. Capítulos III y IV.

2. Estudiar el efecto modulador sobre la microbiota intestinal humana de *Lactobacillus plantarum* IFPL935 debido a su capacidad para metabolizar polifenoles.

Para la consecución de este objetivo se han realizado incubaciones de microbiota colónica con el extracto de polifenoles de vino tinto (Provinols^{TM}) y *L. plantarum* IFPL935 en condiciones estáticas y en un simulador dinámico gastrointestinal. Capítulos IV y V.

3. Analizar el impacto del consumo de vino tinto en la composición de la microbiota oral e intestinal mediante un estudio de intervención en humanos. Capítulos VI y VII.

4. Evaluar el efecto prebiótico de oligosacáridos derivados de lactulosa (OsLu) en la microbiota humana de distintas regiones del colon.

Para la consecución de este objetivo se ha desarrollado un sistema dinámico de simulación gastrointestinal *in vitro* controlado por ordenador, donde se ha evaluado el efecto sobre la microbiota colónica de dietas en las que se han sustituido carbohidratos fácilmente fermentables por OsLu. Capítulos VIII y IX.

III. COMPARATIVE IN VITRO FERMENTATIONS OF CRANBERRY AND GRAPE SEED POLYPHENOLS WITH COLONIC MICROBIOTA

Manuscript accepted in *Food Chemistry*

III.1 ABSTRACT

In this study, we have assessed the phenolic metabolism of a cranberry extract by microbiota obtained from the ascending colon and descending colon compartments of a dynamic gastrointestinal simulator (SHIME). For comparison, parallel fermentations with a grape seed extract were carried out. Extracts were used directly without previous intestinal digestion. Among the 60 phenolic compounds targeted, our results confirmed the formation of phenylacetic, phenylpropionic and benzoic acids as well as phenols such as catechol and its derivatives from the action of colonic microbiota on cranberry polyphenols. Benzoic acid (38.4 µg/mL), 4-hydroxy-5-(3'-hydroxyphenyl)-valeric acid (26.2 µg/mL) and phenylacetic acid (19.5 µg/mL) reached the highest concentrations. Under the same conditions, microbial degradation of grape seed polyphenols took place at lesser extent than for cranberry polyphenols, which was consistent with the more pronounced antimicrobial effect observed for the grape seed polyphenols, particularly against *Bacteroides*, *Prevotella* and *Blautia coccoides-Eubacterium rectale*.

III.2 INTRODUCTION

The first clinical study into the effect of consumption of cranberry (*Vaccinium macrocarpon* or *Oxycoccus macrocarpus*) in treating urinary tract infections (UTIs) dates back to 1966 (Papas, Brusch, & Ceresia, 1966). Since then, most of these studies reported a preventive effect against UTIs (for review, see Vasileiou, Katsargyris, Theocharis, & Giaginis, 2013), although in some particular studies no significant effects were observed (Barbosa-Cesnik, Brown, Buxton, Zhang, De Busscher, & Foxman, 2011; Stapleton et al., 2012).

The red cranberry is rich in several groups of phenolic compounds, especially flavonols (200-400 mg/kg), anthocyanins (136-1710 mg/kg) and proanthocyanidins (PACs) (4188 mg/kg) (Pappas & Schaich, 2009). PACs are oligomers and polymers of flavan-3-ol monomers [mainly (epi)afzelechin, (epi)catechin and (epi)galocatechin] joined by B-type (4β-8 or 4β-6) and additional A-type (2β-O-7 or 2β-O-5) linkages. Oligomeric forms with at least one A-type interflavanic linkage – which awards certain conformational inflexibility to the molecule – predominate in cranberry PACs (Pappas et al., 2009). Besides polyphenols, other phytochemicals occurring in cranberries are terpenes, organic acids, complex carbohydrates, and sugars (Pappas & Schaich, 2009). The beneficial effects of cranberry against UTIs have been attributed, at least partly, to their PAC content and special composition, although which are the most active structures has still not been elucidated (Shmuely, Ofek, Weis, Rones, & Houri-Haddad, 2012; Vasileiou et al., 2013). Other foods only containing B-type PACs, such as grape seeds or apples, lack these preventive properties against UTI exhibited by cranberry. Cranberry A-type PACs have been shown to exhibit uropathogenic *Escherichia coli* (UPEC)-anti-adhesive activity and other activities related to bacterial interaction with host cells to a greater extent than B-type PACs (Feliciano, Meudt, Shanmuganayagam, Krueger & Reed, 2014), but PACs are

unlikely to appear in urine at relevant concentrations as they are poorly absorbed at the intestine level. One leading hypothesis is that cranberry components, and/or their direct metabolites, present in the urine would operate in the phase of bacterial adherence of UPEC to the uroepithelial cells, preventing bacterial colonization and progression of UTIs (Vasileiou et al., 2013). In fact, ex vivo studies confirmed the anti-adhesive activity of urine samples collected from volunteers who consumed cranberry products in comparison to urine samples collected from the placebo group (Howell et al., 2010), suggesting that the compounds responsible for the benefits against UTIs might be products of the metabolism of the cranberry (and/or not-metabolized cranberry components) eliminated in the urine.

With the final aim of looking into the metabolism of cranberry polyphenols in more depth and unravelling the potential mechanisms behind the selective and preventive effects of cranberry consumption against UTIs, we have carried out comparative batch culture fermentations of cranberry and grape seed extracts with colonic microbiota. For these fermentations, human microbiota from the colonic compartments of the dynamic simulator of the human intestinal microbial ecosystem (SHIME) (Molly, van de Woestijne, & Verstraete, 1993) was used. Production of phenolic acids and other related metabolites were monitored over 48 h to assess differences in the metabolic profiles of cranberry and grape seed extracts subjected to the same microbiota and fermentation conditions. Microbial community analyses and microbial metabolic activity (short-chain fatty acids and ammonium production) determinations were also conducted to determine the effects of both extracts on gut microbiota survival.

III.3 MATERIAL AND METHODS

III.3.1 Phenolic standards and extracts

Standards of phenolic compounds were purchased from Sigma-Aldrich Chemical Co (St Louis, MO), Phytolab (Vestenbergsgreuth, Germany) and Extrasynthèse (Genay, France). A commercial cranberry extract was kindly supplied by Triarco Industries Inc. (New Jersey, USA). Total phenolic content of the cranberry extract was 219 mg of gallic acid equivalents/g, as measured by the Folin-Ciocalteu reagent (Merck, Darmstadt, Germany). The cranberry extract contained benzoic acids (9.76 mg/g), hydroxycinnamic acids (11.1 mg/g), flavan-3-ols (2.1 mg/g) and anthocyanins (0.055 mg/g) (sample #18 in Sánchez-Patán, Bartolomé, Martín-Álvarez, Anderson, Howell, & Monagas, 2012b). A commercial grape seed extract (Vitaflavan®) was kindly provided by Les Dérives Resiniques & Terpéniques (DRT), S.A. (France). Total phenolic content of the grape seed extract was 629 mg of gallic acid equivalents/g, as measured by the Folin-Ciocalteu reagent. The grape seed extract mainly contained flavan-3-ols (337 mg/g), including galloylated and non-galloylated forms (Sánchez-Patán et al., 2012a).

III.3.2 *In vitro* batch incubations with human colonic bacteria

In vitro batch incubations were performed by sampling 25 mL of the ascending colon and descending colon compartments (AC and DC, respectively) (~ 8 log copy number/mL) of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This dynamic *in vitro* gastrointestinal model comprises a series of five double-jacketed fermentation vessels simulating the stomach, small intestine and the three-stage large intestine conditions (Molly et al., 1993). The colon compartments contained *in vitro* cultured microbiota that harboured a reproducible human microbial community representative of the *in vivo*

conditions, both in composition and metabolic activity (Van den Abbeele et al., 2010). Following sampling, the colon microbial suspensions (25 mL) were placed into bottles containing cranberry or grape seed extracts (500 mg/L) and were incubated for 48 h at 37 °C. To obtain anaerobic conditions, L-cysteine (0.5 g/L) was added and bottles were closed with butyl rubber stoppers and flushed with N_2 during 15 cycles of 2 min each at 800 mbar over pressure and 900 mbar under pressure. Before starting the incubation, bottles were placed at atmospheric pressure. Samples were taken at 0, 6, 24 and 48 h with a needle that extends beyond the butyl rubber stoppers that seal off the incubation bottles. Upon sampling, the mixture was flushed with N_2 to ensure anaerobic conditions. Samples were immediately stored at −20 °C until further analysis. Just before analysis, samples were defrosted and centrifuged (10000 rpm, for 10 min at 4 ºC); pellets were used for DNA isolation, and supernatants were filtered (0.22 µm) and analysed for phenolic metabolites, short-chain fatty acids and ammonium. For each extract, three independent experiments were carried out.

III.3.3 Targeted analysis of phenolic acids and other metabolites

Phenolic compounds were analysed by a previous UPLC-ESI-MS/MS method (Jiménez-Girón et al. 2013). Filtered supernatants were directly injected into the UPLC equipment. The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA, USA) equipped with a binary pump, an autosampler thermostated at 10 ºC, and a heated column compartment (40 ºC). The column employed was a BEH-C18, 2.1 x 100 mm and 1.7 µm particle size from Waters (Milford, MA, USA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient programme was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total run-time of 18 min. The flow rate was

set constant at 0.5 mL/min and injection volume was 2 µL. The LC effluent was pumped to a Waters Acquity TQD tandem quadrupole mass spectrometer (Milford, MA, USA) equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 ºC; desolvation temperature, 400 ºC; desolvation gas (N2) flow rate, 750 l/h; cone gas (N_2) flow rate, 60 l/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids and valerolactones) were previously reported (Jiménez-Girón et al. 2013). All metabolites were quantified using the calibration curves of their corresponding standards, except for 4-hydroxy-5-(3´,4´-dihydroxyphenyl)-valeric, 4-hydroxy-5-(3´-hydroxyphenyl)-valeric and 4-hydroxy-5-(phenyl)-valeric acids, which were quantified using the calibration curves of 3-(3´,4´-dihydroxyphenyl)-propionic, 3-(3´-hydroxyphenyl)-propionic and propionic acids, respectively. Data acquisition and processing were realized with MassLynx 4.1 software. Injections were carried out in duplicate.

III.3.4 Microbial community analyses

Quantitative PCR (qPCR) on total bacteria and different groups and genera of bacteria (*Lactobacillus, Bifidobacterium, Bacteroides, Prevotella, Enterobacteriaceae, Blautia coccoides-Eubacterium rectale* group, *Clostridium leptum* subgroup and *Ruminococcus*) was performed following the methodology described in Barroso et al. (2013). Briefly, bacterial DNA was extracted using hexadecyltrimethylammonium bromide

(CTAB) buffer and phenol-chloroform-isoamyl alcohol and bead-beating. The DNA was precipitated with polyethelene glycol (PEG-6000), washed in ice-cold 70% ethanol and dried in a Speed-Vac, prior to resuspension in distilled water. The DNA concentration and quality of the samples were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Bacterial numbers in the DNA samples were quantified by qPCR using SYBR green methodology (Kappa Biosystems, Woburn, MA, USA) with the IQ5 Multicolor Real-Time PCR Detection System and data analyses (Bio-Rad Laboratories Inc., Hercules, CA, USA). Primers, amplification conditions and calculation of copy numbers have been detailed previously (Barroso et al., 2013). DNA from *Escherichia coli* DH5α, *L. plantarum* IFPL935, *Bifidobacterium breve* 29M2 and *Bacteroides fragilis* DSM2151 was used for quantification of total bacteria, *Lactobacillus, Bifidobacterium* and *Bacteroides*, respectively. For the rest of the groups analyzed, samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA), as described previously (Barroso et al., 2013). Samples were subjected to DNA extraction and analysed in duplicate.

III.3.5 Analysis of short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA) and ammonium

The SCFA and BCFA were extracted from the samples with diethyl ether, after the addition of 2-methyl hexanoic acid as an internal standard and extracts were analysed as described previously (Alander, De Smet, Nollet, Verstraete, Von Wright, & Mattila-Sandholm, 1999). Briefly, one microlitre of the diethyl ether layer was injected and measured in a Di200 gas chromatograph (GC; Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a capillary-free fatty-acid packed column [EC-1000 Econo-

Cap column (Alltech, Laarne, Belgium), 25 m × 0.53 mm, film thickness 1.2 µm], a flame ionization detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as the carrier gas at a flow rate of 20 mL/min. The column temperature and the temperature of the injector and detector were set at 130 °C and 195 °C respectively. The concentration of SCFA and BCFA was calculated in mg/L. Total SCFA were calculated based on the amounts of acetate, propionate, butyrate, valerate and caproate and BCFA based on the amounts of isobutyrate, isovalerate and isocaproate. Samples were analysed in duplicate.

Ammonium determination was performed as earlier described by Bremner and Keeney (1965). Briefly, ammonium was released from samples as ammonia by the addition of MgO and distillation into a boric acid-indicator solution using an auto distillation Vapodest 30´ (Gerhardt Analytical Systems, Brackley Northants, UK). Ammonia was determined by titration with standard HCl using a 685 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium). Ammonium ion concentration was expressed as mg/L. Samples were analysed in duplicate.

III.3.6 Statistical analysis

Mean values and standard deviations were calculated based on the values for the different variables during the incubation period (phenolic metabolites, microbial groups, SCFA, BCFA, acetate, propionate, butyrate, and ammonium). Concerning data of phenolic metabolites, three-way analysis of variance (ANOVA) was used to test the main effects of three factors studied (time, compartment, and phenolic extract added). Concerning data of fatty acids, ammonium, and microbial groups, one-way ANOVA and posterior least significant difference (LSD) test, and the corresponding non-parametric Kruskal-Wallis test were used

to detect differences along the incubation time, for each compartment and containing either the cranberry or the grape seed extract. The t-test for independent samples, together with the corresponding non-parametric Mann-Whitney test, were used to evaluate differences between the phenolic extracts at a certain time of incubation and in each compartment. The significance level considered was $p = 0.05$. Principal component analysis (PCA), from matrix correlation (where variables were previously standardized using all samples) was used to summarize changes in the concentration of microbial-derived phenolic metabolites. All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984−2006, www.statsoft.com).

III.4 RESULTS

III.4.1 Production of phenolic acids and other metabolites

A total of 27 phenolic compounds were identified and quantified in the supernatants from the incubations of cranberry and grape seed extract with human microbiota obtained from the ascending colon and descending colon compartments (AC and DC, respectively) of the SHIME system (Table III.1). They corresponded to phenylacetic, phenylpropionic, benzoic and cinnamic acids and phenols, and other metabolites exclusively derived from the catabolism of flavan-3-ols, such as phenyl-γ-valerolactones and phenylvaleric acid derivatives. Among them, benzoic acid (38.4 µg/mL), 4-hydroxy-5-(3'-hydroxyphenyl)-valeric acid (26.2 µg/mL, quantified as 4-(3´-hydroxyphenyl)-propionic acid) and phenylacetic acid (19.5 µg/mL) reached the highest concentrations (Table III.1). A three-way ANOVA analysis was applied to assess the main factors (time, phenolic extract and microbiota) that had significant effect ($p < 0.05$) on the concentration of each phenolic compound quantified (Table III.1, Figures III.1 and III.2) (Supporting Information, Figures S1-

S4). A first result to be noted is that incubation of both extracts with AC and DC microbiota led to a general and steady release of phenylacetic and phenylpropionic acids up to 48 h (Figures III.1 and III.2), the time factor being significant in all cases, except for 4-hydroxyacetic and 3-(3'-hydroxyphenyl)-propionic acids (Table III.1). For all these phenolic acids, maximum concentrations were found in incubations with the DC microbiota, either because the initial faecal suspension exhibited higher concentration (e.g. phenylacetic acid), or because they were released to a greater extent in the presence of a phenolic extract [e.g. 3,4-dihydroxyphenylacetic and 3-(3´,4´-dihydroxyphenyl)-propionic acids]. The cranberry extract (500 mg/L) led to a significantly higher production of main metabolites, such as 3,4-dihydroxyphenylacetic, 3-(3´,4´-dihydroxyphenyl)-propionic, 3-(4´-hydroxyphenyl)-propionic and phenylpropionic acids when incubated with both AC and DC microbiota, in comparison to the grape seed extract.

Some benzoic and cinnamic acids were present in the phenolic extract per se, as confirmed from the concentration values at t=0) (Supporting Information, Figures S1 and S2). The grape seed extract contained a relevant quantity of gallic acid (3,4,5-trihydroxybenzoic acid) (Sánchez-Patán et al., 2012a) whereas the cranberry extract was specially rich in benzoic, p-coumaric (4-hydroxycinnamic), protocatechuic (3,4-dihydroxybenzoic) and vanillic (4-hydroxy-3-methoxybenzoic) acids (sample #18 in Sánchez-Patán et al., 2012b). Time and phenolic extract factors resulted as significant factors for the concentrations of all these benzoic and cinnamic acids during the incubation of the two extracts with the two microbiota (Table III.1). Interestingly, gallic acid (grape seed extract) suffered a sharp decrease during the first 6 h of incubation with the AC microbiota, whereas its degradation by the DC was only noticeable after 24 h of incubation (Supporting Information, Figure S1). The same was observed for protocatechuic acid, although to a lesser extent. Similarly, concentrations of cinnamic acids (cranberry extract)

constantly decreased over the first 24 h of incubation in the presence of both AC and DC microbiota (Supporting Information, Figure S2). On the other hand, other compounds, such as 2-hydroxybenzoic acid (salicylic acid) and benzoic acid, increased consistently up to 24 h of incubation with both microbiota, but more so in the case of the cranberry extract (Supporting Information, Figure S1).

Another group of metabolites formed during the incubation of the extracts with the colon microbiota included catechol/pyrocatechol (1,2-dihydroxybenzene) and its derivatives 4-metylcatechol and 4-ethylcatechol. For all of them, the amount released was greater in the presence of the cranberry extract and their incubation followed similar trends (Supporting Information, Figure S3), although only catechol showed significant differences by time and phenolic extract (Table III.1).

Finally, other metabolites derived from the catabolism of flavan-3-ols such as phenyl-γ-valerolactones and phenylvaleric acid derivatives were only detected in the incubations of the grape seed extract with the colonic microbiota (Supporting Information, Figures S4). In the incubations with the DC microbiota, 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol, which is considered a first intermediate in the catabolism pathway of flavan-3-ols, became detectable at 6 h. The phenyl-□-valerolactones and phenylvaleric acids were subsequently formed afterwards, again only in the case of the DC incubations. The three main factors (time, phenolic extract and microbiota) were found significant for most of these compounds (Table III.1).

Principal component analysis (PCA) was performed in order to summarize changes in the concentrations of phenolic acids and other metabolites resulting from the batch culture fermentations of the cranberry and grape seed extracts with the colonic microbiota. Two principal components (PC1 and PC2), which explained 52.3% of the total variance of the data, were obtained. To show the changes over time,

main values of the scores of the triplicate incubations in the different time periods (0, 6, 24 and 48 h) were plotted in the plane defined by the first two principal components (Figure III.3). PC1 (27.3 % of total variance explained) was directly correlated (loadings ≥ 0.7) with 3,4-dihydroxyphenylacetic acid (0.961), 3-(3´,4´-dihydroxyphenyl)-propionic acid (0.941), 3-(4´-hydroxyphenyl)-propionic acid (0.932), phenylpropionic acid (0.917), benzoic acid (0.779) and catechol (0.866), all microbial-derived phenolic metabolites produced during the incubation of phenolic extracts. PC2 (25.0% of total variance explained) was inversely correlated with syringic acid (-0.835), vanillic acid (-0.932), caffeic acid (-0.863), ferulic acid (-0.902), trans-p-coumaric acid (-0.891), and trans-cinnamic acid (-0.919), all initially present in the cranberry extract. Main differences among samples were due to the phenolic extract (cranberry/grape seeds) employed, with low PC2 scores for incubations with the cranberry extract (higher concentrations in benzoic and cinnamic acids). Also, changes in the phenolic content during the incubation time were reflected in both PC1 and PC2, as phenolic metabolites were produced (higher scores for PC1) and initial benzoic and cinnamic acids were degraded (higher scores for PC2). Samples from incubations with AC and DC microbiota were closely located for each phenolic extract and followed a similar trend over time, especially for the cranberry extract. Overall, as time progressed, incubations of the cranberry extract with both AC and DC microbiota led to relatively greater changes in their phenolic profile in comparison to the grape seed extract which suffered phenolic metabolism to a lesser extent.

Table III.1. Range of variation (minimum and maximum values) of the concentration of main phenolic metabolites measured during incubations of the cranberry/grape seeds extract with the microbiota taken from the ascending colon (AC) and descending colon (DC) compartments, and significant main effects of the factors.

			Factors´ main effects		
	Minimum (µg/mL)	Maximum (µg/mL)	Time	Phenolic extract	Microbiota
Phenylacetic acids					
3,4-Dihydroxyphenylacetic acid	0.00	4.55	*	*	*
3-Hydroxyphenylacetic acid	0.00	0.19	*	-	*
4-Hydroxyphenylacetic acid	0.55	5.75	-	-	*
Phenylacetic acid	4.80	19.54	*	-	*
Phenylpropionic acids					
3-(3´,4´-Dihydroxyphenyl)-propionic acid	0.00	5.32	*	*	*
3-(3´-Hydroxyphenyl)-propionic acid	0.00	1.88	-	-	-
3-(4´-Hydroxyphenyl)-propionic acid	0.00	11.32	*	*	-
Phenylpropionic acid	0.00	1.81	*	*	*
Benzoic acids					
Gallic acid	0.00	7.42	*	*	*
Protocatechuic acid	0.04	6.74	*	*	*
Syringic acid	0.00	0.14	*	*	-
Vanillic acid	0.03	2.54	*	*	-
4-Hydroxybenzoic acid	0.13	0.46	*	*	*
2-Hydroxybenzoic acid	0.08	0.47	*	*	*
Benzoic acid	0.22	38.39	*	*	*
Cinnamic acids					
Caffeic acid	0.00	0.92	*	*	-
Ferulic acid	0.00	0.82	*	*	-
Trans-p-coumaric acid	0.00	5.18	*	*	-
Trans-cinnamic acid	0.00	0.80	*	*	-
Phenols					
Catechol/Pyrocatechol	0.00	6.24	*	*	-
4-Metylcatechol	0.00	0.04	-	-	-
4-Ethylcatechol	0.00	0.06	-	-	-
Other metabolites					
1-(3',4'-Dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol	0.00	6.09	*	*	*
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone	0.00	10.22	*	*	*
4-Hydroxy-5-(3´,4´-dihydroxyphenyl)-valeric acid	0.00	15.66	*	*	*
4-Hydroxy-5-(3´-hydroxyphenyl)-valeric acid	0.00	26.20	-	-	-
4-Hydroxy-5-(phenyl)-valeric acid	0.00	15.00	-	-	-

* Statistical significance of the main effects of the factors ($p < 0.05$).

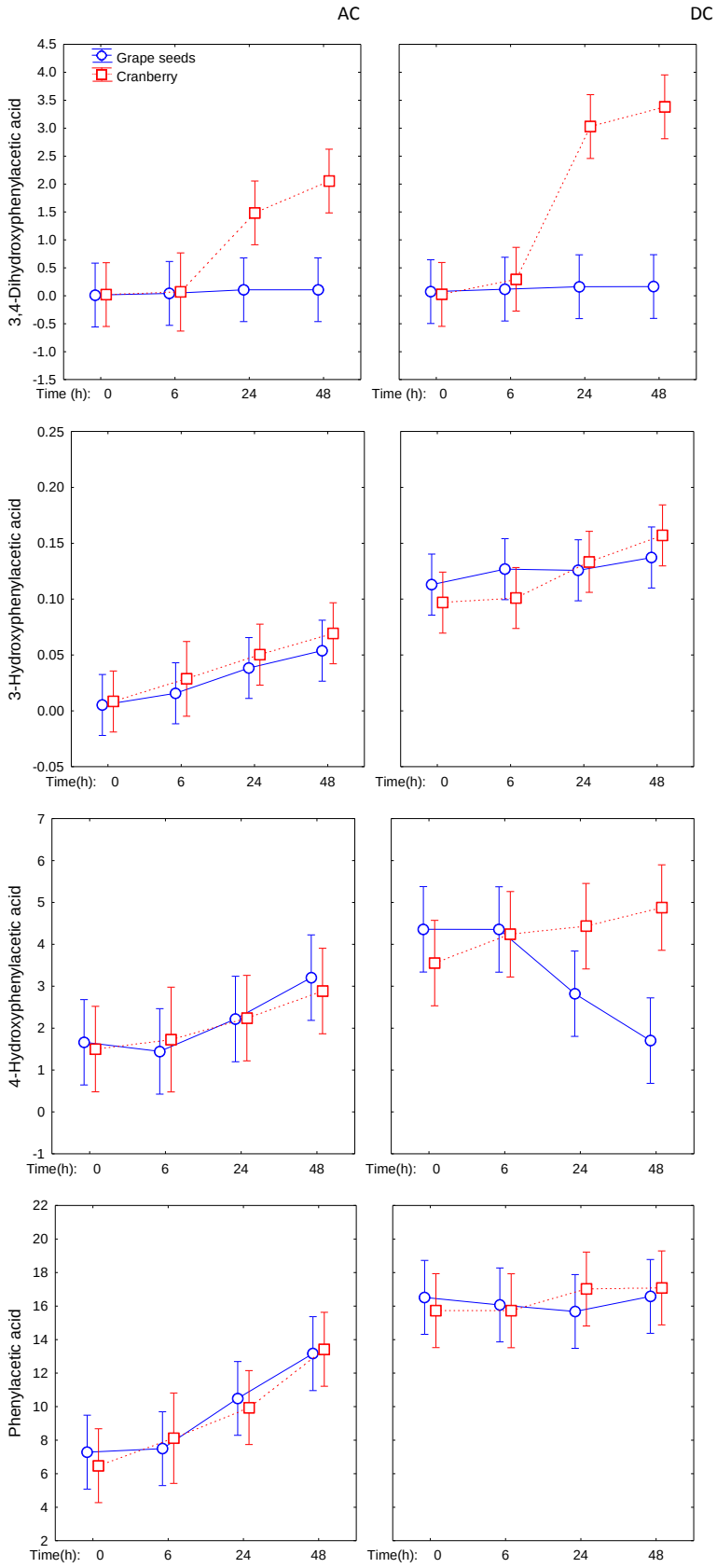


Figure III.1. Mean values of the concentrations of phenylacetic acids (µg/mL) during incubations of phenolic extracts with colonic microbiota: 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid and phenylacetic acid. The error bars are 95% confidence intervals (n=3).

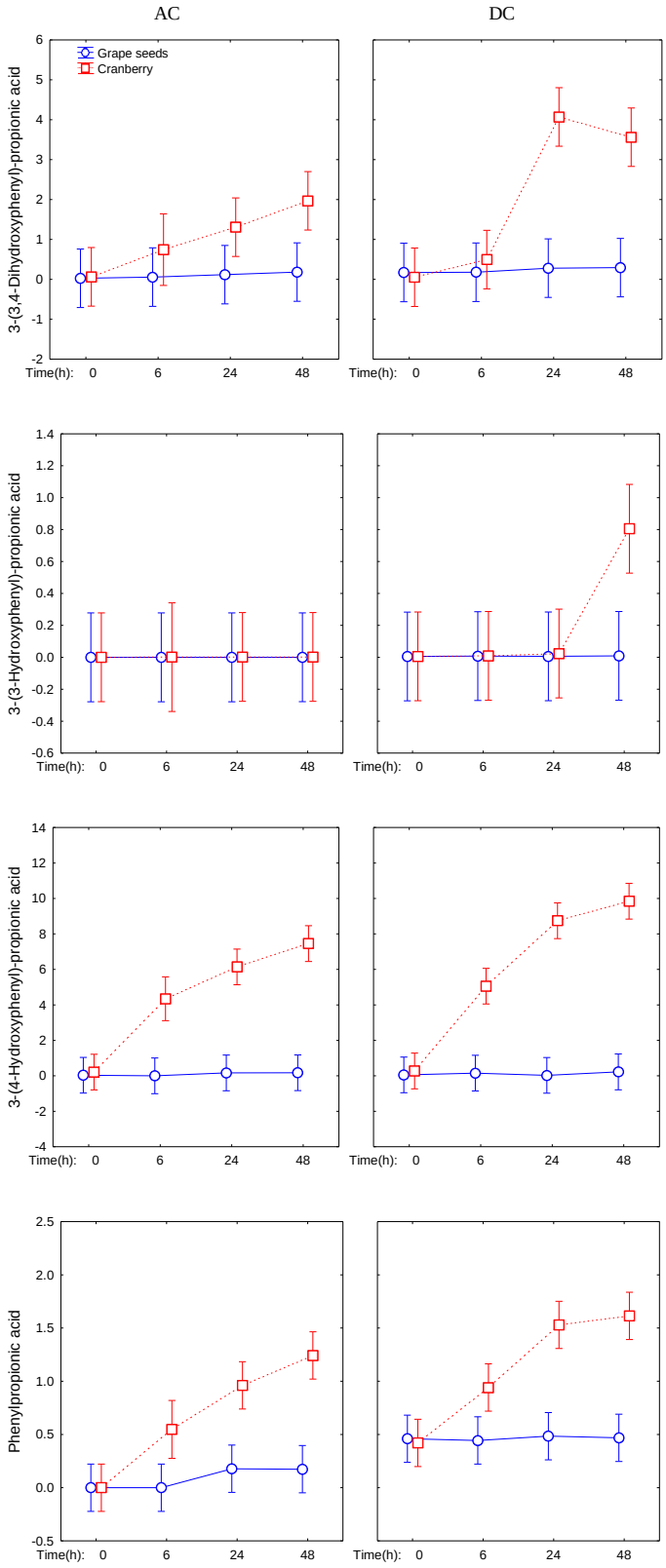


Figure III.2. Mean values of the concentrations of phenylpropionic acids (µg/mL) during incubations of phenolic extracts with colonic microbiota: 3-(3´,4´-dihydroxyphenyl)-propionic acid, 3-(3´-hydroxyphenyl)-propionic acid, 3-(4´-hydroxyphenyl)-propionic acid and phenylpropionic acid. The error bars are 95% confidence intervals (n=3).

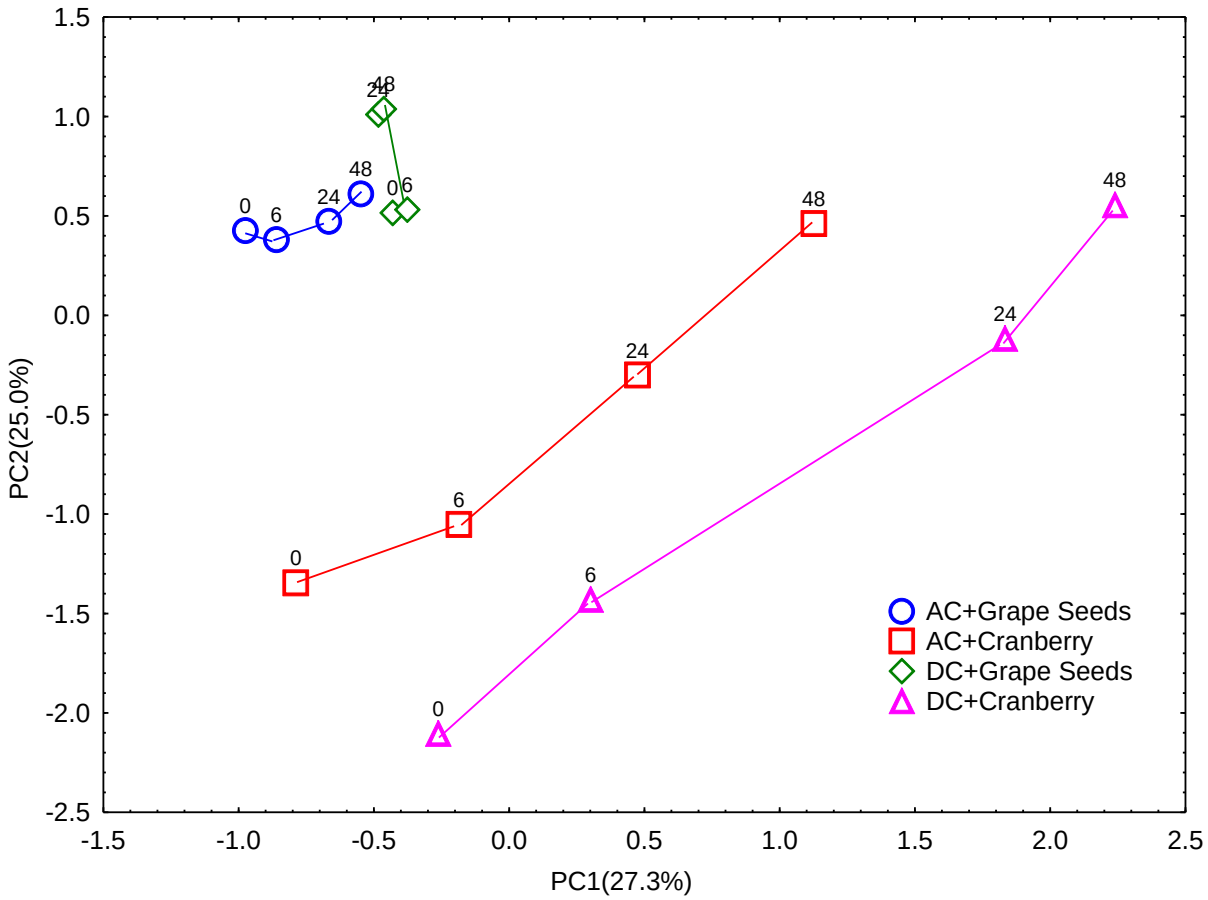


Figure III.3. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulting from a PCA of phenolic compounds from the incubations of phenolic extracts with colonic microbiota at different times (0, 6, 24 and 48 h).

III.4.2 Changes in microbial counts and metabolism

The impact of the cranberry and grape seed extracts on the AC and DC microbiota from the SHIME was analysed by qPCR, targeting total bacteria and specific phylogenetic groups (Table III.2). Significant differences ($p < 0.05$) in microbiological counts were observed between the AC and DC batches at the incubation onset; *Lactobacillus*, *Bacteroides*, *Prevotella* and *Enterobacteriaceae* being representative of the AC microbiota, whereas *C. leptum* and *Ruminococcus* prevailed in the DC microbiota. Similar results were obtained with the nonparametric Mann-Whitney test. During the batch incubations, counts from all the analysed bacterial groups generally decreased over time ($p < 0.05$). The reduction of microbial counts could be partially associated with the limitation of substrates during the batch incubations. Moreover, differences in the evolution of microbial groups were observed, depending on the polyphenolic extract analysed. The decrease of *Enterobacteriaceae* counts was faster when the samples were incubated with cranberry rather than with grape seed, showing a decrease in 1.5 log copy numbers/mL after 24 h of incubation in both the AC and DC batches added with the cranberry extract (Table III.2). However, for most of the microbial groups assayed, the highest decrease in counts was observed in the DC batches after 48 h of incubation with the grape seed extract, showing an average decrease in counts of 2 log copy numbers/mL for *Bifidobacterium*, *Lactobacillus*, *Enterobacteriaceae*, *C. leptum* and *Ruminococcus* and of 3 log copy numbers/mL for *Bacteroides*, *Prevotella* and *B. coccoides-E. rectale*. On the other hand, the decrease of the microbial groups in the AC batches was similar during the incubation with both phenolic extracts, showing *Prevotella* as having the highest decrease in counts (about 4 log copy numbers/mL after 48 h of incubation; Table III.2). The same significant differences during the batch incubations indicated in the Table III.2 were obtained using the nonparametric Kruskal-Wallis test, although some for a value of $p < 0.08$.

Table III.2. Means ± standard deviation values of the quantitative PCR data (log copy number/mL) for the microbial groups analyzed during incubations of the cranberry/grape seeds extract with the microbiota taken from the ascending colon (AC) and descending colon (DC) compartments.

Bacterial group		Compartment	Incubation time		
			0 h	24 h	48 h
Total bacteria	Cranberry	AC	8.71 ± 0.09c	7.72 ± 0.11b	6.71 ± 0.45a
		DC	8.32 ± 0.33b	7.17 ± 0.40a	6.56 ± 0.50a
	Grape seed	AC	8.61 ± 0.24b	8.38 ± 0.34b	6.64 ± 0.02a
		DC	8.18 ± 0.41b	*7.06 ± 0.51b	5.34 ± 0.80a
Lactobacillus	Cranberry	AC	7.37 ± 0.06c	6.74 ± 0.06b	5.80 ± 0.44a
		DC	*6.78 ± 0.22b	5.68 ± 0.51a	5.54 ± 0.63a
	Grape seed	AC	7.40 ± 0.10c	6.92 ± 0.17b	5.79 ± 0.08a
		DC	*6.80 ± 0.29c	*5.55 ± 0.50b	4.52 ± 0.57a
Bifidobacterium	Cranberry	AC	7.53 ± 0.03b	7.11 ± 0.01b	5.96 ± 0.38a
		DC	6.90 ± 0.45b	6.62 ± 0.65ab	5.83 ± 0.32a
	Grape seed	AC	7.51 ± 0.14b	7.25 ± 0.18b	6.13 ± 0.16a
		DC	6.78 ± 0.59b	5.92 ± 0.82ab	4.84 ± 0.82a
Bacteroides	Cranberry	AC	7.43 ± 0.02c	6.82 ± 0.31b	5.74 ± 0.27a
		DC	*6.87 ± 0.06b	*5.99 ± 0.25ab	5.64 ± 0.89a
	Grape seed	AC	7.51 ± 0.19c	7.22 ± 0.06b	5.62 ± 0.04a
		DC	*7.04 ± 0.15c	*5.81 ± 0.17b	4.01 ± 0.97a
Prevotella	Cranberry	AC	7.74 ± 0.24c	5.38 ± 0.33b	4.15 ± 0.40a
		DC	*6.15 ± 0.20b	*4.44 ± 0.35a	3.98 ± 0.50a
	Grape seed	AC	7.87 ± 0.09c	6.56 ± 0.28b	4.34 ± 0.10a
		DC	*6.41 ± 0.39c	*4.23 ± 0.55b	*3.04 ± 0.57a
Enterobacteriaceae	Cranberry	AC	8.56 ± 0.13c	7.13 ± 0.17b	5.78 ± 0.45a
		DC	*7.52 ± 0.30b	*6.01 ± 0.52a	5.56 ± 0.43a
	Grape seed	AC	8.83 ± 0.03c	8.18 ± 0.11b	6.16 ± 0.26a
		DC	*7.52 ± 0.43c	*6.38 ± 0.47b	5.15 ± 0.72a
B. coccoides-E. rectale	Cranberry	AC	7.34 ± 0.02b	6.97 ± 0.21b	5.82 ± 0.49a
		DC	6.94 ± 0.19c	6.21 ± 0.43b	5.45 ± 0.36a
	Grape seed	AC	7.27 ± 0.03b	7.01 ± 0.13b	5.96 ± 0.19a
		DC	6.95 ± 0.30b	*5.70 ± 0.62b	*4.23 ± 0.85a
Clostridium leptum	Cranberry	AC	5.00 ± 0.06b	5.21 ± 0.44b	4.08 ± 0.61a
		DC	5.98 ± 0.47b	5.11 ± 0.61ab	4.37 ± 0.29a
	Grape seed	AC	5.09 ± 0.25b	5.10 ± 0.26b	3.79 ± 0.30a
		DC	*6.09 ± 0.20b	4.59 ± 0.63a	3.70 ± 0.92a
Ruminococcus	Cranberry	AC	3.98 ± 0.06c	3.57 ± 0.05b	3.01 ± 0.32a
		DC	*5.24 ± 0.19b	4.53 ± 0.42ab	*4.15 ± 0.42a
	Grape seed	AC	3.97 ± 0.04b	3.66 ± 0.04b	2.24 ± 0.68a
		DC	*5.28 ± 0.20c	4.17 ± 0.46b	3.11 ± 0.69a

a-c For a given microbial group analyzed, different letters denote significant differences ($p < 0.05$) from LSD test during the incubation time (for a given compartment) with the grape seed and cranberry extracts.

* Denotes differences ($p < 0.05$) between the two compartments for a given incubation time and each extract.

The effect of the cranberry and grape seed extracts on the fermentative and proteolytic activities of the AC and DC microbial groups was evaluated by the formation of short-chain (SCFA) and branched-chain (BCFA) fatty acids, and ammonium (Table III.3). The initial content of SCFA was higher in the samples originated from the DC vessel of the SHIME than those from the AC compartment, whereas the BCFA and ammonium contents showed no significant differences within colonic compartments. Acetic, propionic and butyric acids represented more of the 90% of the SCFA analysed while isovaleric and isobutyrate acids were the predominant BCFA. During incubation, an increase in the formation of SCFA was only observed in the incubations with the AC microbiota, but no significant differences were observed in the effect of the added polyphenolic extract. The AC batches were characterized as containing the major proportion of saccharolytic bacteria (*Bacteroides*, *Lactobacillus* and *Prevotella*; Table III.2), which are mainly responsible of the formation of SCFA. Likewise, the formation of BCFA and ammonium only increased during incubation of the phenolic extracts with the AC microbiota (Table III.3). The results obtained with the nonparametric Kruskal-Wallis test matched those of the one-way ANOVA, although four comparisons were statistically significant but for a value of $p < 0.08$.

Table III.3. Mean ± standard deviation data for acetate, propionate, butyrate, total SCFA, BCFA and ammonium during incubation of the ascending colon (AC) and descending colon (DC) compartments with the cranberry and grape seed extracts.

			Incubation time		
Compound		Compartment	0 h*	24 h	48 h
Acetate	Cranberry	AC	1877[a] ± 281a	1923 ± 317a	2141 ± 49a
		DC	2497 ± 254a	2586 ± 380a	2370 ± 344a
	Grape seed	AC	1877 ± 282a	1963 ± 213a	2011 ± 70a
		DC	2497 ± 254a	2387 ± 602a	2351 ± 226a
Propionate	Cranberry	AC	761 ± 66a	946 ± 139ab	1014 ± 64b
		DC	993 ± 96a	1006 ± 108a	929 ± 124a
	Grape seed	AC	761 ± 66a	936 ± 124b	975 ± 55b
		DC	993 ± 96a	976 ± 198a	944 ± 39a
Butyrate	Cranberry	AC	399 ± 71a	582 ± 66b	640 ± 58b
		DC	481 ± 12a	486 ± 25a	529 ± 75a
	Grape seed	AC	399 ± 71a	579 ± 57b	604 ± 45b
		DC	481 ± 12a	458 ± 72a	*482 ± 18a
SCFA	Cranberry	AC	3244 ± 453a	3740 ± 568a	4100 ± 156a
		DC	4526 ± 397a	4656 ± 533a	4580 ± 633a
	Grape seed	AC	3244 ± 453a	3762 ± 392a	3905 ± 163a
		DC	4526 ± 397a	4385 ± 946a	4438 ± 390a
BCFA	Cranberry	AC	255 ± 64a	315 ± 35a	329 ± 22a
		DC	356 ± 21a	356 ± 27a	363 ± 47a
	Grape seed	AC	255 ± 64a	319 ± 24a	330 ± 13a
		DC	356 ± 21a	371 ± 54a	367 ± 19a
Ammonium	Cranberry	AC	238 ± 60a	305 ± 19ab	321 ± 30b
		DC	381 ± 8a	348 ± 36a	369 ± 36a
	Grape seed	AC	238 ± 60a	296 ± 6ab	327 ± 11b
		DC	381 ± 8a	*380 ± 24a	358 ± 38a

Data are expressed as means ± standard deviation. For a given compound analysed, different lower-case letters, in the same row (compartment), denote significant differences ($P < 0.05$) along the incubation time; and * denotes differences between the two compartments ($P < 0.05$), for each incubation time, and each extract. aData at time 0 h correspond to the values analysed from the AC and DC compartment samples before addition of the phenolic extracts.

III.5 DISCUSSION

Consumption of cranberry is widely recommended as a prophylaxis against UTIs, although the mechanisms behind these preventive effects are still poorly understood. These preventive effects associated with cranberry consumption may be due to the combination of different polyphenols rather than to the action of an individual or single phenolic group alone (Wang, Zuo, Vinson, & Y. Deng, 2012). Therefore, experimentation with cranberry extracts/products rather than with specific cranberry components (i.e. A-type proanthocyanidins), seems an accurate approach to studying their impact on human health. To our knowledge, this is the first *in vitro* study of the degradation of cranberry polyphenols by human microbiota, evaluating both phenolic metabolism by colonic microbiota and the modulation of microbiota by cranberry polyphenols and/or their metabolites. Our results confirmed the formation of phenylacetic (3,4-dihydroxy-, 3-hydroxy-, 4-hydroxy-, and phenylacetic), phenyl-propionic [3-(3´,4´-dihydroxy-phenyl)-, 3-(3´-hydroxy-phenyl)-, 3-(4´-hydroxy-phenyl)- and 3-(phenyl)propionic acid] and benzoic (3,4-dihydroxy-, 4-hydroxy-, 2-hydroxy- and benzoic acid) acids, as well as phenols such as catechol and its derivatives (4-methy- and 4-ethyl) derived from the action of colon microbiota on cranberry polyphenols. Therefore, it would be expected that these metabolites could be absorbed from the large intestine and be further detected in the plasma and urine after the ingestion of cranberry products. Effectively, Wang et al. (2012) found a significant increase in the content of 4-hydroxyphenylacetic, 4-hydroxybenzoic acid, 2-hydroxybenzoic and benzoic acids, among other metabolites, in urine samples of healthy subjects after a 3-week administration of cranberry juice. In the same way, Khanal, Howard & Prior (2014) found that supplementation of a high-fructose diet with cranberry significantly increased the urinary excretion of 3,4-dihydroxyphenylacetic, 3-hydroxyphenylacetic, 3-(3´-

hydroxphenyl)-propionic and 3,4-dihydroxybenzoic, among other phenolic acids, in rats.

Another contribution of this study is the comparison between the microbial degradation sequence of cranberry and grape seed polyphenols. Grape seed extracts, rich in B-type proanthocyanidins, have been widely used for the study of the microbial metabolism of flavan-3-ols and their concurrent impact on gut microbiota. In a previous study using the same grape seed extract (Sánchez-Patán et al., 2012a), maximum formation of intermediate metabolites, such as 5-(3´,4-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3´,4´-dihydroxyphenyl)-valeric acid, and of several phenolic acids, including 3-(3,4-dihydroxyphenyl)-propionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxymandelic acid, and gallic acid was observed at 5–10 h of incubation with faecal microbiota; later phases of the incubations (10–48 h) showed the appearance of mono- and non-hydroxylated forms of previous metabolites by dehydroxylation reactions. Formation of all these metabolites was also detected in the present study, but to a lesser extent, which was attributed to differences in the microbiota and in the incubation conditions used. In any case, in comparison to grape seed polyphenols, microbial degradation of cranberry polyphenols led to a different phenolic metabolic fingerprint, characterized by a relatively higher production of 3,4-dihydroxyphenylacetic, 3-(3´,4´-dihydroxyphenyl)-propionic, 3-(4´-hydroxyphenyl)-propionic and phenylpropionic acids (Table III.1, Figures III.1 and III.2) (Supporting Information, Figures S1-S4). When all metabolites detected were considered in a statistical multivariable analysis (PCA), we concluded that the extent of microbial degradation was greater in the case of cranberry polyphenols (Figure III.3). These comparative results concerning phenolic metabolism were associated with changes in microbial counts and metabolic activity for both cranberry and grape seed extracts. The lower extent of phenolic metabolism was related with a higher decrease in microbial counts observed in the DC

batches incubated with the grape seed extract (Table III.2), and agrees with previous results that demonstrated a certain antimicrobial capacity for this same grape seed extract (Cueva et al. 2013; Tabasco et al., 2011). A comparison of the effect of grape seed and cranberry extracts in the growth of pure cultures of lactic acid bacteria revealed a higher reduction in growth parameters caused by the incubation with grape seed extract (Tabasco et al., 2011) than with cranberry extract (Sánchez-Patán et al., 2012c), showing procyanidin B2 (B-type linkage) to be a higher inhibitory capacity than procyanidin A2 (A-type linkage) (Sánchez-Patán et al., 2012c). Monomeric flavan-3-ols and B-type procyanidin dimers have been described as showing a wide spectrum and higher antimicrobial activity in comparison with other polyphenols (Daglia, 2012).

During the incubation of the colonic microbiota with the phenolic extracts, the groups most affected were *Bacteroides*, *Prevotella* and B. coccoides-E. rectale. In a previous study we observed that *Bacteroides* and the B. coccoides-E. rectale group were the most inhibited groups after feeding the SHIME colonic microbiota with red wine polyphenols (Barroso et al., 2014). *In vitro* fermentation studies with faecal microbiota have also revealed that (+)-catechin and (epi)gallocatechins were able to inhibit Clostridium and *Bacteroides* (Tzounis et al., 2008). On the other hand, the higher counts of microbial groups registered in the AC batches corresponded with a time-dependent increase of the fermentative and proteolytic metabolism observed in these batches (Table III.3). *Bacteroides* and *Lactobacillus* are characterized by exhibiting saccharolytic and proteolytic activities (Ravcheev, Godzik, Osterman, & Rodionov, 2013; Davila et al., 2013).

Additionally, this study confirms differences in the metabolic activity of colon microbiota from different regions (i.e, ascending and descending colon). Formation of phenolic acids from the microbial conversion of cranberry and grape seed polyphenols was higher with the DC

microbiota, which is in agreement with previous studies (Barroso et al. 2013). Within the microbial groups assayed, the clostridial cluster IV, represented by *C. leptum* and *Ruminococcus*, prevailed in the DC batches. So far, only a few bacterial species, some of them belonging to the class *Clostridiales*, such as *Eubacterium* sp. and *Flavonifractor* sp., and *Eggerthella* spp., have been reported to be able to initiate the metabolism of flavanol-3-ols (Wang et al. 2001; Kutschera, Engst, Blaut, & Braune, 2011; Jin & Hattori 2012). Although batch incubations with faecal microbiota are limited by substrate depletion and the accumulation of the end products, they are appropriate for comparison of the microbial consequences of exposure to different sources or doses of compounds, such as the cranberry and grape seed extracts used in this study.

In summary, this study reports for the first time the formation of phenolic acids and other metabolites after *in vitro* incubations of cranberry polyphenols with colon microbiota, to a relatively greater extent in comparison to polyphenols from other sources (i.e., grape seeds). These microbial-derived metabolites may play a key role in explaining the preventive effects of cranberry consumption against UTIs. Recently, the anti-adhesive activity against uropathogenic *E. coli* by some microbial-derived metabolites, such as catechol, benzoic acid, vanillic acid, phenylacetic acid and 3,4-dihydroxyphenylacetic acid, has been proven in cultures of epithelial bladder cells (González de Llano, Esteban-Fernández, Sánchez-Patán, Martín-Álvarez, Moreno-Arribas, & Bartolomé, 2014).

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III.6 REFERENCES

Alander, M., De Smet, I., Nollet, L., Verstraete, W., Von Wright, A., & Mattila-Sandholm, T. (1999). The effect of probiotic strains on the microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). International Journal of Food Microbiology, 46, 71-79.

Barbosa-Cesnik, C., Brown, M. B., Buxton, M., Zhang, L., DeBusscher, & J., Foxman, B. (2011). Cranberry juice fails to prevent recurrent urinary tract infection: results from a randomized placebo-controlled trial. Clinical Infectious Diseases, 52, 23-30.

Barroso, E., Sanchez-Patan, F., Martin-Alvarez, P. J., Bartolome, B., Moreno-Arribas, M. V., Pelaez, C. et al. (2013). *Lactobacillus plantarum* IFPL935 favors the initial metabolism of red wine polyphenols when added to a colonic microbiota. Journal of Agricultural and Food Chemistry, 61, (42) 10163-10172.

Barroso, E., Van de Wiele, T., Jiménez-Girón, A., Muñoz-González, I., Martin-Alvarez, P. J., Moreno-Arribas, M. V. et al. (2014) *Lactobacillus plantarum* IFPL935 impacts colonic metabolism in a simulator of the human gut microbiota during feeding with red wine Polyphenols. Applied Microbiology and Biotechnology, 98, 6805-6815.

Bremner, J. M. & Keeney, R. D. (1965). Steam distillation methods for determination of ammonium, nitrate and nitrite. Analytica Chimica Acta, 32, 485−495

Cueva, C., Sanchez-Patan, F., Monagas, M., Walton, G. E., Gibson, G. R., Martin-Alvarez, P. J. et al., (2013). *In vitro* fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: changes in microbial groups and phenolic metabolites. FEMS Microbial Ecology, 83(3) 792-805.

Daglia, M. (2012). Polyphenols as antimicrobial agents. Current Opinion in Biotechnology, 23, 174–181.

Davila, A. M., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P.H., Sanz, Y. et al. (2013). Intestinal luminal nitrogen metabolism: role of the gut microbiota and consequences for the host. British Journal of Nutrition, 110, 625-631.

Feliciano, R. P., Meudt, J.J., Shanmuganayagam, D., Krueger, C. G., & Reed, J. D. (2014). Ratio of "A-type" to "B-type" Proanthocyanidin Interflavan Bonds Affects Extra-intestinal Pathogenic *Escherichia coli* Invasion of Gut Epithelial Cells. Journal of Agricultural and Food Chemistry, 62(18), 3919-3925.

González de Llano, D., Esteban-Fernández, A., Sánchez-Patán, F., Martín-Álvarez, J. P., Moreno-Arribas, M. V., & Bartolomé, B. (2014). Antiadhesive activity of phenolic compounds and their microbial-derived metabolites against uropathogenic *Escherichia coli* in bladder epithelial cell cultures. Submitted.

Howell, A. B., Botto, H., Combescure, C., Blanc-Potard, A. B., Gausa, L., Matsumoto, T., et al. (2010). Dosage Effect on Uropathogenic *Escherichia coli* Anti-Adhesion Activity in Urine Following Consumption of Cranberry Powder Standardized for Proanthocyanidin Content: a Multicentric Randomized Double Blind Study. BMC Infectious Diseases, 10, 94.

Jin, J. S. & Hattori, M. (2012). Isolation and Characterization of a Human Intestinal Bacterium Eggerthella sp CAT-1 Capable of Cleaving the C-Ring of (+)-Catechin and (-)-Epicatechin, Followed by p-Dehydroxylation of the B-Ring. Biological & Pharmaceutical Bulletin, 35(12) 2252-2256.

Jiménez-Girón, A., Queipo-Ortuño, M. I., Boto-Ordoñez, M., Muñoz-González, I., Sanchez-Patan, F., Monagas, M. et al. (2013). Comparative study of microbial-derived phenolic metabolites in human feces after intake of gin, red wine, and dealcoholized red wine. Journal of Agricultural and Food Chemistry, 61, 3909–3915.

Khanal, R., Howard, L. R., & Prior, R. L. (2014). Urinary Excretion of Phenolic Acids in Rats Fed Cranberry, Blueberry, or Black Raspberry Powder. Journal of Agricultural and Food Chemistry, 62(18) 3987-3996.

Kutschera, M., Engst, W., Blaut, M., & Braune, A. (2011). Isolation of catechin-converting human intestinal bacteria. Journal of Applied Microbiology, 111(1) 165-175.

Molly, K., van de Woestijne, M., & Verstraete, W. (1993). Development of a 5-step multichamber reactor as a simulation of the human intestinal microbial ecosystem. Applied Microbiology Biotechnology, 39, 254–258.

Papas, P. N., Brusch, C. A., & Ceresia, G.C. (1966). Cranberry juice in the treatment of urinary tract infections. Southwest Medical, 47, 17-20.

Pappas, E., Schaich, K. M. (2009). Phytochemicals of cranberries and cranberry products: characterization, potential health effects and processing stability. Critical Reviews in Food Science and Nutrition, 49, 741-781.

Ravcheev, D. A., Godzik, A., Osterman, A. L., & Rodionov, D. A. (2013). Polysaccharides utilization in human gut bacterium *Bacteroides thetaiotaomicron*: comparative genomics reconstruction of metabolic and regulatory networks. BMC Genomics, 14, 873.

Sánchez-Patán, F., Bartolomé, B., Martín-Álvarez, J. P., Anderson, M., Howell, A., & Monagas, M. (2012b). Comprehensive assessment of

the quality of commercial cranberry products. Phenolic characterization and *in vitro* bioactivity. Journal of Agricultural and Food Chemistry, 60, 3396-3408.

Sánchez-Patán, F., Cueva, C., Monagas, M., Walton, G. E., Gibson, G. R., Martín-Álvarez, J. P., et al. (2012a). Gut microbial catabolism of grape seed flavan-3-ols by human faecal microbiota. targeted analysis of precursor compounds, intermediate metabolites and end products. Food Chemistry, 131, 337-347.

Sánchez-Patán, F., Tabasco, R., Monagas, M., Requena, T., Peláez, C., Moreno-Arribas M. V. et al. (2012c). Capability of *Lactobacillus plantarum* IFPL935 to catabolize flavan-3-ol compounds and complex phenolic extracts. Journal of Agricultural and Food Chemistry, 60, 7142−7151.

Shmuely, H., Ofek, I., Weis, E. I., Rones, Z., & Houri-Haddad, Y. (2012). Cranberry components for the therapy of infectious disease. Current Opinion in Biotechnology, 23, 148–152.

Stapleton, A. E., Dziura, J., Hooton, T. M., Cox, M. E., Yarova-Yarovaya Y., Chen, S., et al. (2012). Recurrent urinary tract infection and urinary *Escherichia coli* in women ingesting cranberry juice daily: a randomized controlled trial. Mayo Clinic Proceedings, 87, 143-50.

Tabasco, R., Sánchez-Patán, F., Monagas, M., Bartolomé, B., Moreno-Arribas, M. V., Peláez, C. et al. (2011). Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: Resistance and metabolism. Food Microbiology, 28, 1345−1352.

Tzounis, X., Vulevic, J., Kuhnle, G. G. C., George, T., Leonczak, J., Gibson, G. R. et al. (2008). Flavanol monomer-induced changes to the human faecal microflora. British Journal of Nutrition, 99(4) 782-792.

Van den Abbeele, P., Grootaert, C., Marzorati, M., Possemiers, S., Verstraete, W., Gérard, P. et al. (2010). Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for *Bacteroidetes* and *Clostridium* cluster IX. Applied and Environmental Microbiology, 76, 5237–5246.

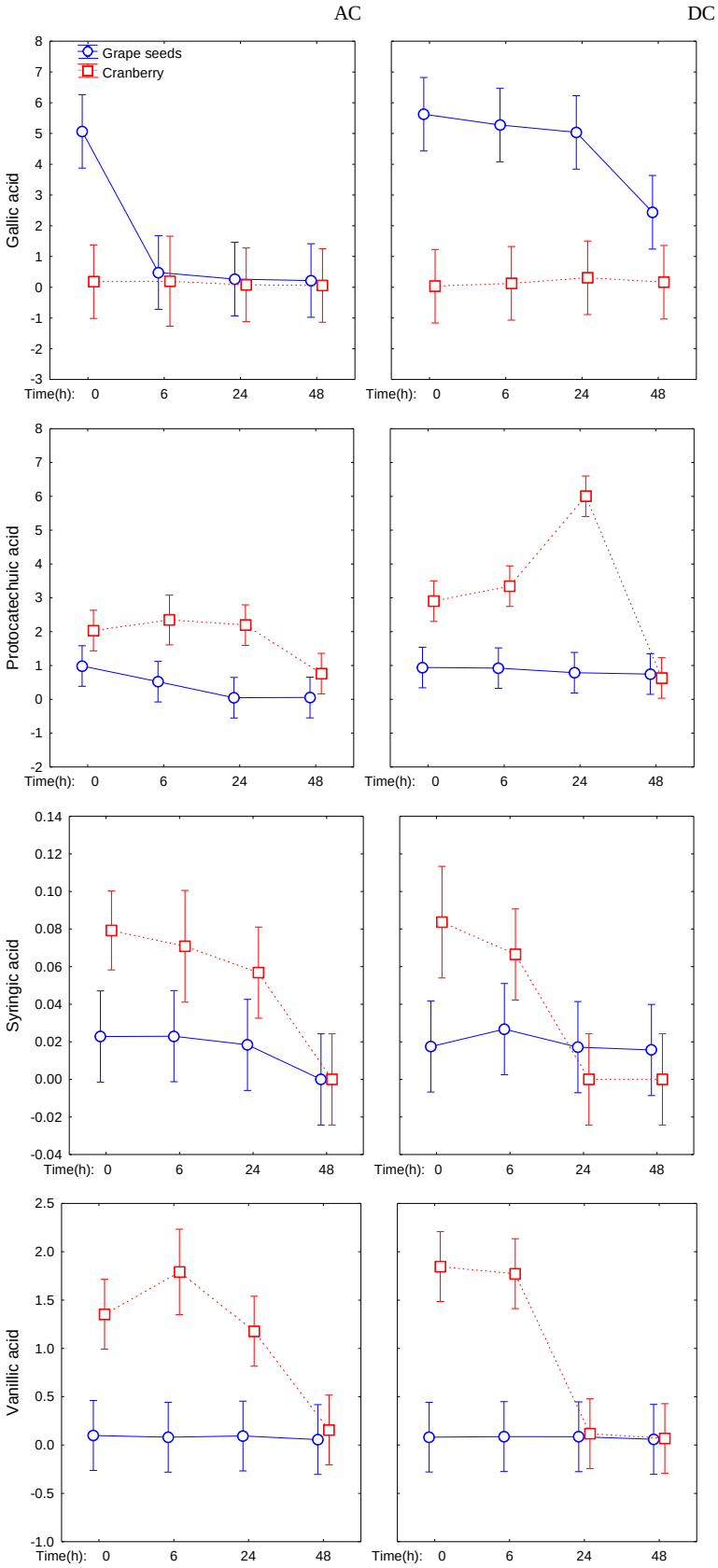
Vasileiou, I., Katsargyris, A., Theocharis, S., & Giaginis, C. (2013) Current clinical status on the preventive effects of cranberry consumption against urinary tract infections. Nutrition Research, 595-607.

Wang, C., Fang, C., Chen, N., Liu, S. S., Yu, P. H., Wu, T. Y., et al. (2012). Cranberry-containing products for prevention of urinary tract infections in susceptible populations: a systematic review and meta-analysis of randomized controlled trials. Archives of Internal Medicine, 172, 988-996.

Wang, L. Q., Meselhy, M. R., Li, Y., Nakamura, N., Min, B. S., Qin, G. W. et al. (2001) The heterocyclic ring fission and dehydroxylation of catechins and related compounds by Eubacterium sp strain SDG-2, a human intestinal. Chemical & Pharmaceutical Bulletin, 49(12) 1640-1643.

Wang, C., Zuo, Y, Vinson, J. A., & Deng, Y. (2012). Absorption and excretion of cranberry-derived phenolics in humans, Food Chemistry, 132, 1420-1428.

III.7 SUPPORTING INFORMATION



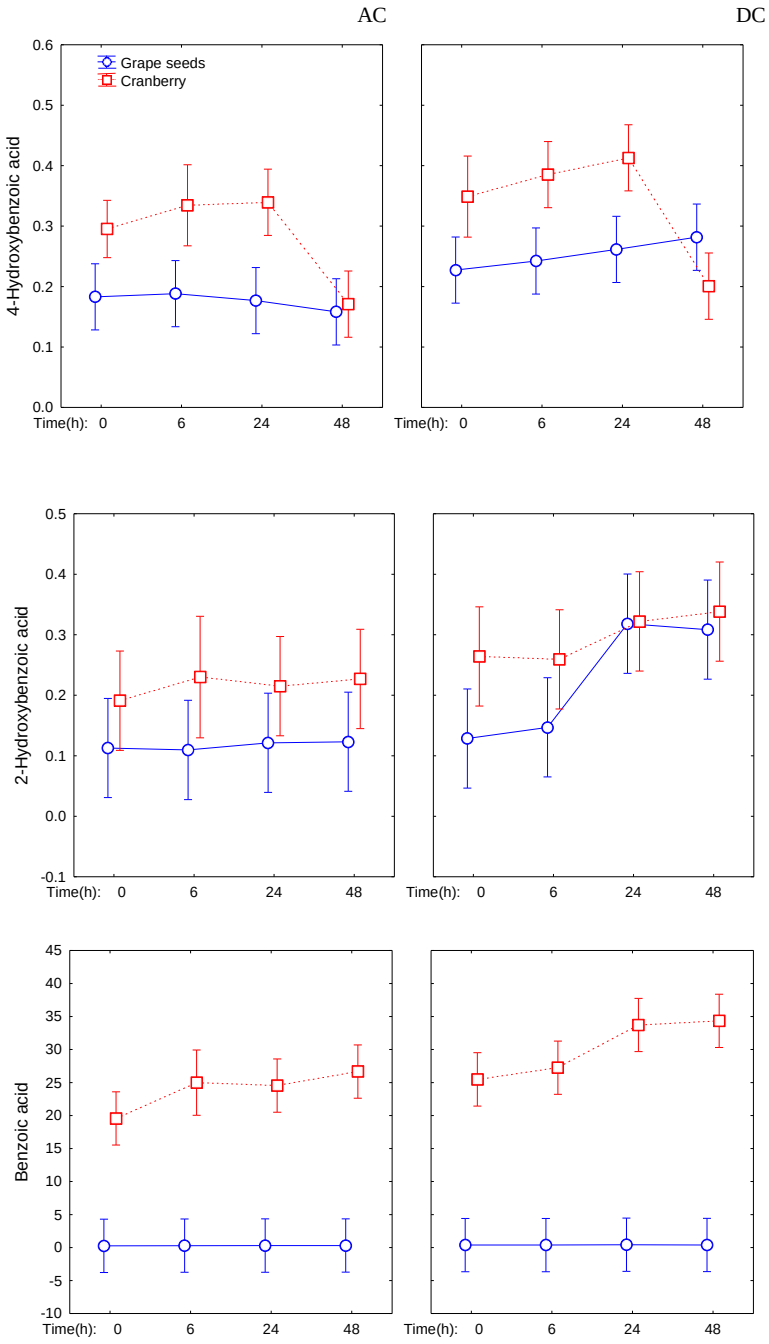


Figure S1. Mean values of the concentrations of benzoic acids (µg/mL) during incubations of phenolic extracts with colonic microbiota: gallic acid, protocatechuic acid, syringic acid, vanillic acid, 4-hydroxybenzoic acid, 2-hydroxybenzoic acid and benzoic acid. The error bars are 95% confidence intervals (n=3).

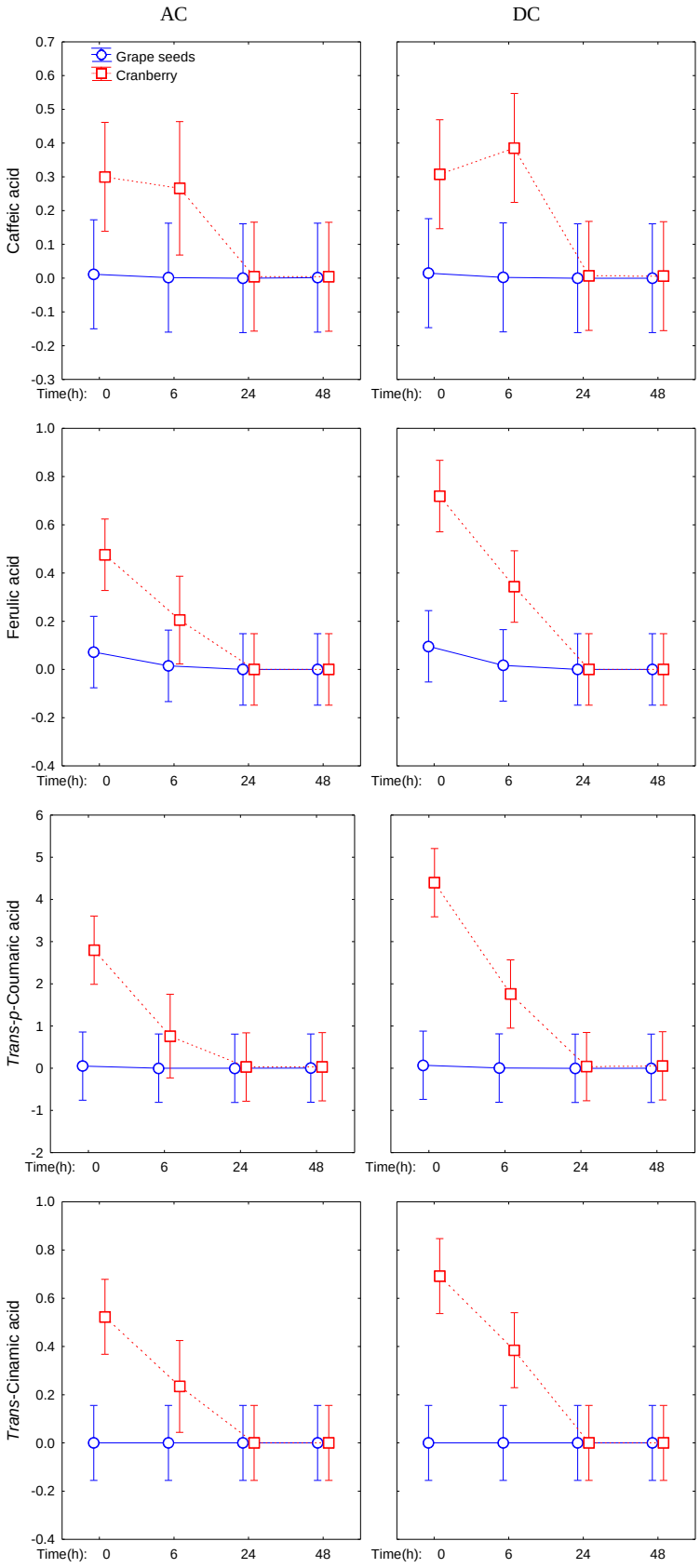


Figure S2. Mean values of the concentrations of cinnamic acids (µg/mL) during incubations of phenolic extracts with colonic microbiota: caffeic acid, ferulic acid, trans-p-coumaric acid and trans-cinnamic acid. The error bars are 95% confidence intervals (n=3).

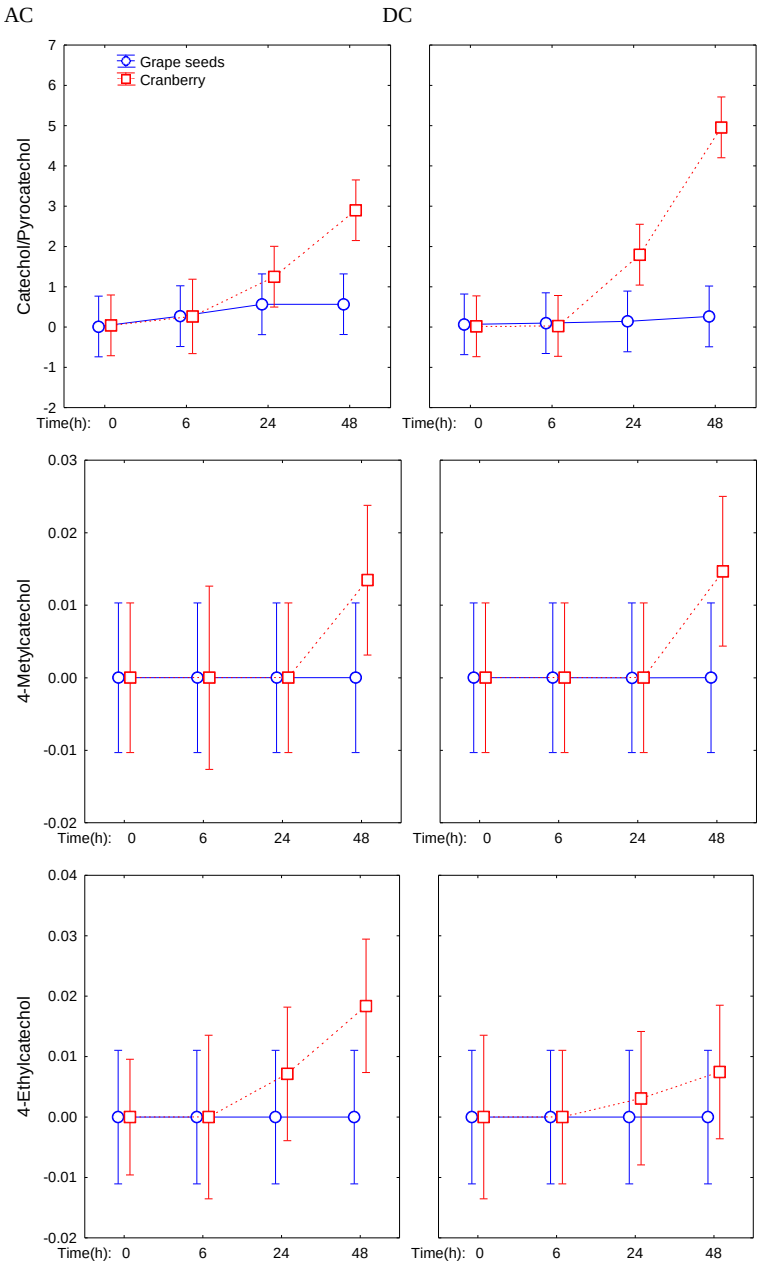


Figure S3. Mean values of the concentrations of phenols (µg/mL) during incubations of phenolic extracts with colonic microbiota: catechol/pyrocatechol, 4-metylcatechol and 4-ethylcatechol. The error bars are 95% confidence intervals (n=3).

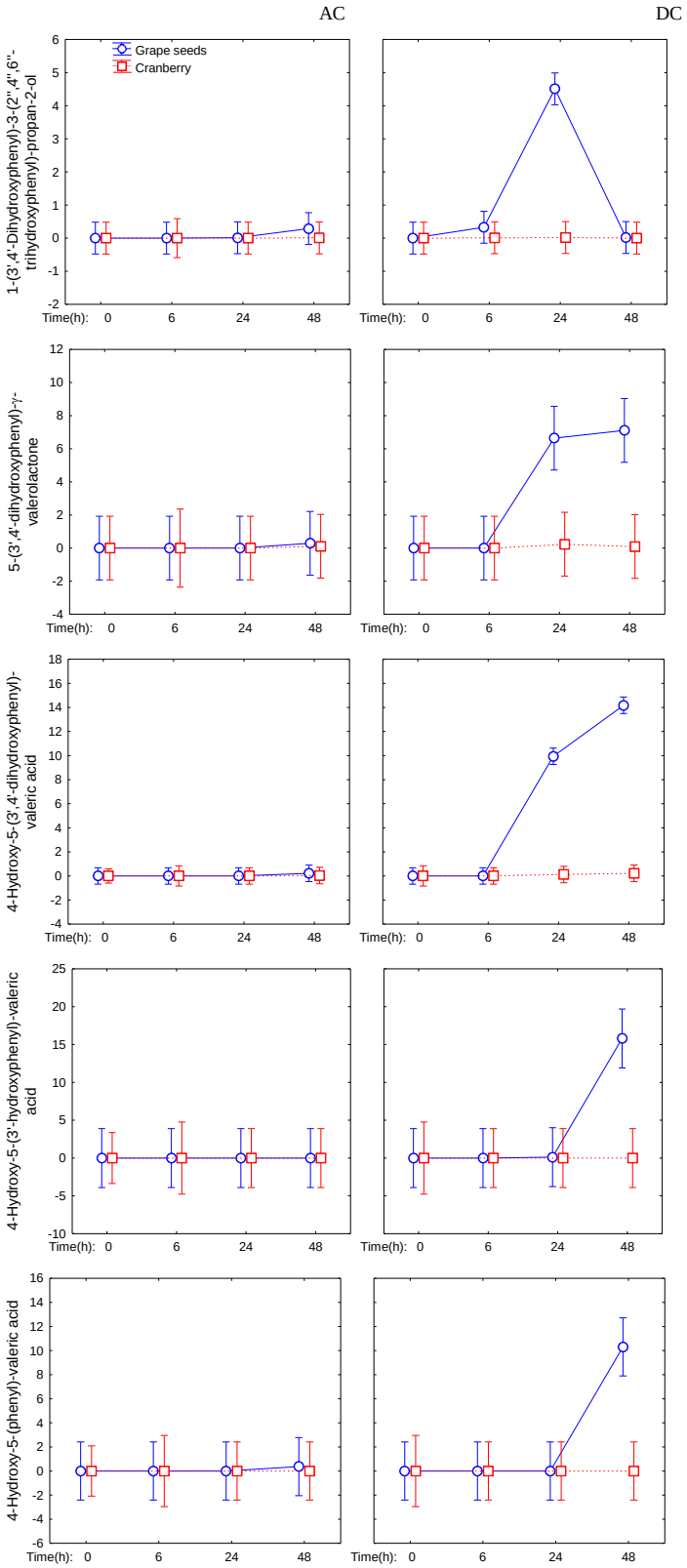


Figure S4. Mean values of the concentrations of other metabolites (µg/mL) during incubations of phenolic extracts with colonic microbiota: 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol,5-(3',4' dihydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(3´,4´-dihydroxyphenyl)-valeric acid, 4-hydroxy-5-(3´-hydroxyphenyl)-valeric acid and 4-hydroxy-5-(phenyl) valeric acid. The error bars are 95% confidence intervals (n=3).

IV. *LACTOBACILLUS PLANTARUM* IFPL935 INITIATES THE METABOLISM OF A POLYPHENOLIC RED WINE EXTRACT IN A COMPLEX INTESTINAL HUMAN MICROBIOTA

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IV.1 ABSTRACT

This work aimed to unravel the role of the potential probiotic *Lactobacillus plantarum* IFPL935 strain in the colonic metabolism of a polyphenolic red wine extract, when added to a complex human intestinal microbiota from the dynamic Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The microbial metabolism of phenolic compounds, microbial community changes along with fermentative and proteolytic activities was monitored. The results showed that *L. plantarum* IFPL935 significantly increased the concentration of the initial microbial ring-fission catabolite of catechins and procyanidins, diphenylpropanol and similarly, 4-hydroxy-5-(3'-hydroxyphenyl)-valeric acid production. Furthermore, the addition of this strain raised an increase of butyrate production. In summary, the data highlighted that *L. plantarum* IFPL935 may have an impact on the bioavailability of these dietary polyphenols and therefore on its biological *in vivo* effects. Also, *L. plantarum* IFPL935 may possible influence SCFAs production, and in particular butyrate, which have numerous documented effects promoting bowel large function.

IV.2 INTRODUCTION

There is increased epidemiological evidence that associates a moderate consumption of red wine to several health benefits in humans such as protection against cardiovascular and neurodegenerative diseases and certain types of cancer including colon, basal cell, ovarian, and prostate carcinoma (Del Rio et al., 2013). This protective effect has been mainly linked to the presence of polyphenol compounds in wine. Polyphenols are secondary plant metabolites widely diverse and abundantly present in our diet in different food and beverages. Flavonoids are the most abundant, and among the most bioactive polyphenols present in red wine, mainly including flavan-3-ols (also known as flavanols), flavonols and anthocyanins (Waterhouse et al,2002). Unlike other classes of flavonoids, which exist in plants primarily in glucoside forms, flavanols are usually present in the aglycone form as monomers or oligomers (condensed tannins or proanthocyanidins) or esterified with gallic acid giving rise to epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). Nevertheless, it is becoming clear that their resulting *in vivo* bioactivity is dependent on its bioavailability, absorption, and metabolism.

Gut microbiota may play a crucial role in the potential health effects of the polyphenols (Crozier, 2009). Absorption of polyphenols from the upper intestinal tract is highly variable although it is established that many of them are poorly absorbed. Bioavailability of flavan-3-ols is largely influenced by their degree of polymerization; while monomers are readily absorbed in the small intestine, oligomers and polymers need to be biotransformed by the colonic microbiota before absorption (Hackman et al., 2008). The human intestinal microbiota is a highly complex and dynamic ecosystem that harbors over a thousand different strains. In the colon, bacterial numbers can reach 100 trillion bacteria and such large numbers have enormous and diverse metabolic activity with significant

implication for plant polyphenol metabolism (van Duynhoven et al., 2011). This metabolic activity, which exceeds that of the liver in a factor of 100, is responsible for the major transformations of polyphenols in compounds that may have higher bioactivity and/or biological significance than their precursors (Possemiers et al., 2011). Microbial enzymes can hydrolyze glycosides, glucuronides, sulfates, amides, esters, and lactones and are able to break down the polyphenolic skeleton and perform reactions of reduction, decarboxylation, demethylation, and dehydroxylation (Aura 2008).

Parallel to this microbial metabolism, polyphenols could also modify the intestinal bacterial population composition and/or activity, thus establishing a bi-directional interaction between intestinal microbiota and dietary polyphenols (Selma et al., 2009; Requena et al., 2010). Related to this, batch fermentations performed with human feces inoculated with catechin have shown to have a positively effect on the growth of the *Blautia coccoides-Eubacterium rectale* group and *Bifidobacterium*, while inhibiting the growth of the *Clostridium histolyticum* group (Tzounis et al., 2008). *In vivo* studies conducted in animals have shown an increase in *Lactobacillus* and *Bifidobacterium* species following administration of red wine-polyphenols while a decrease in *Clostridium* and *Bacteroides* was reported (Dolara et al., 2005). Recently, a pilot human intervention study (n=8) has shown a significant increase in the number of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *B. coccoides–E. rectale* groups in fecal samples after the consumption of red wine polyphenol for 4 weeks in comparison to baseline (Queipo-Ortuño et al., 2012).

In previous studies, we have reported that some strains of *Lactobacillus plantarum*, *Lactobacillus* casei and *Lactobacillus* delbrueckii subsp. bulgaricus were able to grow in the presence of a flavan-3-ol extract from grape seeds. In addition, *L. plantarum* IFPL935 was also

capable of metabolizing these polyphenolic extracts through galloyl-esterase, decarboxylase and benzyl alcohol dehydrogenase enzyme activities leading to the formation of gallic acid, pyrogallol and catechol, respectively (Tabasco et al.,2011). Interestingly, *L. plantarum* IFPL935 showed the potential to cleave the heterocyclic ring of monomeric flavan-3-ols, giving rise to 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (i.e. diphenylpropanol), which is the first metabolite in the microbial catabolic pathway of flavan-3-ols (Sánchez-Patán et al., 2012). This activity has only been reported in a few intestinal bacteria to date (Wang et al., 2011; Kutschera et al., 2011).

The aim of this work was to unravel the role of the potential probiotic *L. plantarum* IFPL935 strain in the colonic metabolism of polyphenols carrying out *in vitro* batch incubations containing a human intestinal microbiota from the colonic compartments of the dynamic Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and a commercial polyphenolic red wine extract. The study was undertaken by monitoring changes in the main microbial groups along with metabolic activity (short-chain fatty acids and ammonium production) and polyphenolic catabolism analyses.

IV.3 MATERIALS AND METHODS

IV.3.1 Bacterial strains, media and phenolic extract

L. plantarum IFPL935 isolated from raw milk cheeses (Fontecha, et al., 1990) (CIAL Collection, CSIC, Spain) was grown at 37 °C in MRS broth (Pronadisa, Madrid, Spain). Growth of bacterial cultures was routinely monitored by measuring the optical density at 600 nm (OD600).

A commercial alcohol-free red wine extract, ProvinolsTM, which contains at least 95% of polyphenols was used (Safic-Alcan

Especialidades, Barcelona, Spain). As indicated by the supplier, 100 mg of Provinols™ correspond to the polyphenol content of one glass of red wine (125 mL). Phenolic composition of this red wine extract was reported before (Sánchez-Patán et al., 2012), being flavan-3-ols and anthocyanins as the main phenolic compounds.

IV.3.2 *In vitro* batch incubations with intestinal bacteria

In vitro batch incubations were performed by sampling 25 mL of the colon ascendens, colon descendens and effluent compartments (AC, DC and EC, respectively) of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) (Molly et al., 1993). This dynamic *in vitro* gastrointestinal model comprises a series of five double-jacketed fermentation vessels simulating the stomach, small intestine and the three-stage large intestine conditions. The colon compartments contained *in vitro* cultured microbiota that were isolated from human feces and harbored a reproducible microbial community representative of the *in vivo* conditions both in composition and metabolic activity (Van den Abbeele et al., 2010). Detailed information about the SHIME system, the SHIME feed and its *in vivo* validation can be found (Molly et al., 1993; Molly et al., 1994; Possemiers et al., 2006). Following sampling, the colon microbial suspensions (25 mL) were placed into penicillin bottles containing Provinols™ (500 mg/L) or Provinols™ (500 mg/L) plus *L. plantarum* IFPL935 (10^7 ufc/mL) and were incubated for 48 h at 37 °C. To obtain anaerobic conditions, L-cystein (0.5 g/L) was added and bottles were closed with butyl rubber stoppers and flushed with N_2 during 15 cycles of 2 min each at 800 mbar overpressure and 900 mbar underpressure. Before starting the incubation, bottles were placed on atmospheric pressure. Samples were taken at 0, 6, 24 and 48 h with a needle that extends beyond the butyl rubber stoppers that seal off the incubation bottles. Upon sampling, the mixture was flushed with N_2 to ensure anaerobic conditions. Samples were immediately stored at −20 °C until

further analysis. Two independent experiments, each of them analyzed in triplicate, were carried out.

IV.3.3 Microbial community analyses

Quantitative PCR (qPCR) on total bacteria and different groups and genera of bacteria (Table III.1) was performed to study the effect of the incubation of the intestinal bacteria with the wine phenolic extract. Genomic DNA was extracted from samples (1 mL) by a method based on the protocols described previously (Griffiths et al., 2000; Kowalchuk et al., 1998). Extractions were performed by the addition of 0.5 mL of hexadecyltrimethylammonium bromide (CTAB) extraction buffer (pH 8.0) and 0.5 mL of phenol-chloroform-isoamyl alcohol (25:24:1). Samples were lysed with glass beads (150-212 µm-diameter) by using a FastPrep equipment (BIO 101, Savant Instruments, Holbrook, NY) for 30 s (3 times) at a machine speed setting of 4.5 m/s. The aqueous phase containing nucleic acids was separated by centrifugation (3,000 × g) for 5 min at 4°C and mixed with an equal volume of chloroform-isoamyl alcohol (24:1) followed by centrifugation (16,000 × g) for 5 min at 4 °C. Total nucleic acids were subsequently precipitated from the extracted aqueous layer with 2 volumes of polyethelene glycol PEG-6000 (Merck, Hohenbrunn, Germany) for 2 h at room temperature, followed by centrifugation (18,000 × g) at 4°C for 10 min. Pelleted nucleic acids were washed in ice cold 70% (vol/vol) ethanol and dried in a Speed-Vac (SPD 111 V; Savant Instruments), prior to resuspension in 100 µL of distilled water.

Triplicate samples of 10-fold diluted genomic DNA were analyzed for total bacteria, *Enterobacteriaceae*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, the specific phylogenetic groups *Blautia coccoides-Eubacterium rectale* Cluster XIVa, *Ruminococcus* Cluster IV, *Clostridium leptum* subgroup specific cluster IV and the gene encoding

butyryl-CoA:acetate CoA transferase (BcoAT). Assays were performed using SYBR green methodology (Kappa Biosystems, Woburn, MA, USA) with the IQ5 Multicolor Real-Time PCR Detection System (Biorad) and data analyses were performed with iQ5 Optical System Software Version 1.1. Target microbial groups and functional genes, primers, amplicon size, annealing temperature, pure bacteria culture or clone for standard curves and references are listed in Table IV.1. When samples were quantified using standards derived from one clone, each specific target sequence was cloned separately using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The recombinant vector was transformed into chemically competent *E. coli* DH5α cells. Transformed colonies were picked and processed for plasmid isolation. Plasmid purification was done using a Plasmid Mini kit (Qiagen, Hilden, Germany). The presence of the insert in the recombinant clones was confirmed by sequence analysis. Linearized plasmid was quantified using a spectrophotometer and copy numbers were calculated for all standards by the following formula (Godornes et al., 2007):

$$\text{Number of copies/µl} = \frac{6.022 \times 10^{23}\ (\text{molecules/mole}) \times \text{DNA concentrations (g/µL)}}{\text{Number of base pairs} \times 660\ \text{daltons}}$$

Table IV.1. Primer sets used in this study for quantitative PCR

Target gene/group	Primer name	Primer sequence 5'-3'	Amplicon size	Annealing temperature	Standard	Reference
Total bacteria	968 F	AACGCGAAGAACCTTAC	489	55	*Escherichia coli* DH5α	Nübel et al., 1996
	1401 R	CGGTGTGTACAAGACCC				Nübel et al., 1996
Lactobacillus	LactoF	TGGAAACAGRTGCTAATACCG	192	55	*Lactobacillus plantarum* IFPL 935	Byun et al., 2004
	LactoR	GTCCATTGTGGAAGATTCCC				Byun et al., 2004
Bifidobacterium	G Bifid F	CTCCTGGAAACGGGTGG	593	55	*Bifidobacterium breve* 29M2	Matsuki et al., 2002
	G Bifid R	GGTGTTCTTCCCGATATCTACA				Matsuki et al., 2002
Bacteroides	Bac303F	GAAGGTCCCCCACATTG	103	60	*Bacteroides fragilis* DSM2151	Bartosch et al., 2004
	Bfr-Fmrev	CGCKACTTGGCTGGTTCAG				Ramirez-Farias et al., 2009
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	513	55	Clone	Matsuki et al., 2002
	g-Prevo-R	GGTCGGGTTGCAGACC				Matsuki et al., 2002
Enterobacteriaceae	F-Ent	ATGGCTGTCGTCAGCTCGT	385	58	Clone	Leser et al., 2002
	R-Ent	CCTACTTCTTTTGCAACCCACTC				Sghir et al., 2000
Clostridium coccoides-Eubacterium rectale group	CcocErec-F	CGGTACCTGACTAAGAAGC	429	55	Clone	Rinttilä et al., 2004
(Cluster XIVa)	CcocErec-R	AGTTTYATTCTTGCGAACG				Rinttilä et al., 2004
Clostridium leptum subgroup-specific	sg-Clept F	GCACAAGCAGTGGAGT	239	55	Clone	Matsuki et al., 2004
(Cluster IV)	sg-Clept R3	CTTCCTCCGTTTTGTCAA				Matsuki et al., 2004
Ruminococcus	Rflbr730F	GGCGGCYTRCTGGGCTTT	157	60	Clone	Ramirez-Farias et al., 2009
(Cluster IV)	Clep866mR	CCAGGTGGATWACTTATTGTGTTAA				Ramirez-Farias et al., 2009
Butyryl-CoA:acetate-CoA transferase gene	BCoATscrF	GCIGAICATTTCACITGGAAYWSITGGCAYATG	557	53	Clone	Louis et a., 2007
	BCoATscrR	CCTGCCTTTGCAATRTCIACRAANGC				Louis et a., 2007

IV.3.4 Short-chain fatty acids (SCFAs) and ammonium determination analyses

The SCFAs were extracted from the samples with diethyl ether, after the addition of 2-methyl hexanoic acid as an internal standard and extracts were analysed as described previously (Alander et al., 1999). Briefly, one microliter of the diethyl ether layer was injected and measured in a Di200 gas chromatograph (GC; Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a capillary free fatty acid packed column [EC-1000 Econo-Cap column (Alltech, Laarne, Belgium), 25 m × 0.53 mm; film thickness 1.2 µm], a flame ionization detector and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as carrier gas at a flow rate of 20 mL/min. The column temperature and the temperature of the injector and detector were set at 130 °C and 195 °C respectively. The concentration of SCFAs was calculated in mg/L.

Ammonium determination was performed as described (Bremner et al.,1965). Chiefly, ammonium from the samples (1 mL) was released as ammonia by addition of magnesium oxide (MgO) using an autodistillation Vapodest 30´ (Gerhardt Analytical Systems, Brackley Northants, UK); thus, ammonia was separated by steam distillation, collected in boric acid-indicator solution and determined by titration with standard acid using a 685 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium). Ammonium ion concentration was expressed as mmol/L.

IV.3.5 Targeted analysis of phenolic metabolites

Phenolic metabolites were analysed by a previously-reported UPLC-ESI-MS/MS method (Sánchez-Patán et al., 2011). The liquid chromatographic system was a Waters Aquity UPLC (Milford, MA) equipped with a binary pump, autosampler thermostated at 10 ºC and a

heated column compartment (40 ºC). The column employed was a BEH-C18, 2.1 x 100 mm and 1.7 µm particle size from Waters (Milford, MA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1% B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total run time of 18 min. The flow rate was set constant at 0.5 mL min-1 and injection volume was 2 µL.

The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 ºC; desolvation temperature, 400 ºC; desolvation gas (N_2) flow rate, 750 L/h; cone gas (N_2) flow rate, 60 L/h. The ESI was operated in negative mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound, and using external calibration curves. Data of the MS/MS parameters (MRM transitions, cone voltages and collision energies) for phenolic metabolites (phenols, mandelic acids, benzoic acids, hippuric acids, phenylacetic acids, phenylpropionic acids, valeric acids, cinnamic acids, valerolactones and other metabolites) (n=60) and for flavan-3-ols (monomers, procyanidin dimers, procyanidin trimers and gallates) were previously optimized (Jiménez-Girón et al., 2013). Quantification was made on the basis of pure standards [from Sigma-Aldrich Chemical Co. (St. Louis, MO), Phytolab (Vestenbergsgreuth, Germany) and Extrasynthèse (Genay, France)] except for valeric acids (4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric, 4-hydroxy-5-(3'-hydroxyphenyl)-valeric and 4-hydroxy-5-(phenyl)-valeric acids) that were quantified using the calibration curves of 3-(3,4-dihydroxyphenyl)-propionic, 3-(3-hydroxyphenyl)-propionic acids and propionic acids, respectively. Injections were carried

out in duplicate. Data acquisition and processing was realized with MassLynx 4.1 software.

IV.3.6 Statistical analysis

Mean values, standard deviations and correlation coefficients were calculated on the basis of normalized values for the different variables during the incubation period (microbial groups, SCFAs, acetate, propionate, butyrate, ammonium and phenolic metabolites). Analysis of variance (ANOVA) was used for multiple comparisons of the different variables taken into account different factors (time, compartments, and addition of *L. plantarum* IFPL935) ($P < 0.05$). Principal Component Analysis (PCA), from standardized variables, was used to summarize changes in the concentration of microbial-derived phenolic metabolites. All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984−2006, www.statsoft.com).

IV.4 RESULTS

IV.4.1 Microbial community analyses

Quantitative PCR (qPCR) was used to analyze the microbial community composition by targeting general bacteria and specific phylogenetic and functional groups (Table IV.2). The microbiological results of the batches at the incubation onset reproduced the variations in bacterial populations of the SHIME colonic compartments. *Bacteroides*, *Bifidobacterium* and *Prevotella* were predominant in the AC (ascending colon) batches whereas *C. leptum* and *Ruminococcus* prevailed in DC (descending colon) incubations. Microbiota found in EC (effluent compartment) batches, more representative of the fecal microbiota, was more similar to the DC batches than to the AC ones. Besides, qPCR data showed that counts of all microbial groups assessed decreased along the

incubation with the polyphenolic extract. Reduction of microbial counts was also associated to the fact that batch culture incubations used in this study were static and closed systems in which the substrate was limited. These *in vitro* systems, however, have been stated as useful for short time course experiments (Gibson et al., 2000).

Inoculation of the colonic microbial batches with *L. plantarum* IFPL935 did not increase the total bacteria nor the *Lactobacillus* counts, although a slightly increase of *Lactobacillus* numbers was observed during incubation of the DC and EC batches inoculated with *L. plantarum* IFPL935 (Table IV.2). Similarly, DC batches inoculated with *L. plantarum* IFPL935 showed significantly higher counts of *Bifidobacterium*, *Bacteroides* and *Ruminococcus* after 48 h incubation than DC batches without IFPL935. Nevertheless, for the other microbial groups analyzed, no significant differences were found in the batches inoculated with a colon region-specific microbiota when IFPL935 was added.

In addition to *Clostridium* clusters IV and XIVa counts, a molecular approach based on the enumeration of the butyryl-CoA:acetate CoA transferase (BCoAT) gene was used for estimating the number of butyrate-producing bacteria. Data analysis showed that BCoAT gene copy number decreased along the incubation in all batches representing the distinct colon region microbiota, along with the reduction of bacteria populations also found at 48 h (Table IV.2). Relating these findings to the microbial groups analyzed, it was found a significant ($P < 0.05$) correlation between this functional gene and the presence of *C. leptum* (r= 0.91), *B. coccoides-E. rectale* group (r= 0.84) and to a lesser extent to *Ruminococcus* (r= 0.79).

Table IV.2. Quantitative-PCR data (log copy number/mL)

Bacterial Group	Assays	Compartment	Incubation Time		
			0 h	24 h	48 h
Total bacteria	Provinols	AC	8.53 (0.32) $^{B\ c}$	7.81 (0.10) $^{BC\ b}$	6.76 (0.33) $^{B\ a}$
		DC	7.80 (0.48) $^{A\ b}$	7.08 (0.37) $^{A\ b}$	5.68 (0.67) $^{A\ a}$
		EC	8.52 (0.31) $^{B\ b}$	7.95 (0.31) $^{C\ b}$	7.02 (0.67) $^{B\ a}$
	Provinols + *L. plantarum* IFPL93	AC	8.24 (0.36) $^{B\ c}$	7.36 (0.41) $^{AB\ b}$	6.49 (0.02) $^{B\ a}$
		DC	8.11 (0.54) $^{AB\ b}$	6.97 (0.48) $^{A\ a}$	6.46 (0.59) $^{AB\ a}$
		EC	8.48 (0.38) $^{B\ b}$	8.07 (0.47) $^{C\ b}$	6.90 (0.64) $^{B\ a}$
Lactobacillus	Provinols	AC	7.25 (0.33) $^{B\ c}$	6.63 (0.01) $^{CD\ b}$	5.65 (0.37) $^{B\ a}$
		DC	6.49 (0.53) $^{A\ b}$	5.79 (0.40) $^{A\ b}$	4.57 (0.50) $^{A\ a}$
		EC	7.05 (0.14) $^{AB\ b}$	6.64 (0.30) $^{CD\ b}$	5.66 (0.70) $^{B\ a}$
	Provinols + *L. plantarum* IFPL93	AC	7.05 (0.44) $^{AB\ c}$	6.43 (0.40) $^{BC\ b}$	5.67 (0.05) $^{B\ a}$
		DC	6.88 (0.58) $^{AB\ b}$	5.90 (0.51) $^{AB\ a}$	5.52 (0.51) $^{B\ a}$
		EC	7.31 (0.20) $^{B\ b}$	7.12 (0.41) $^{D\ b}$	6.07 (0.63) $^{B\ a}$
Bifidobacterium	Provinols	AC	7.31 (0.32) $^{B\ b}$	7.09 (0.10) $^{B\ b}$	5.94 (0.20) $^{B\ a}$
		DC	6.59 (0.71) $^{A\ b}$	6.31 (0.60) $^{A\ b}$	4.94 (0.69) $^{A\ a}$
		EC	6.66 (0.15) $^{A\ b}$	6.37 (0.27) $^{AB\ b}$	5.46 (0.75) $^{AB\ a}$
	Provinols + *L. plantarum* IFPL93	AC	7.10 (0.43) $^{B\ b}$	6.71 (0.57) $^{AB\ b}$	5.70 (0.03) $^{B\ a}$
		DC	6.90 (0.80) $^{A\ b}$	6.19 (0.79) $^{A\ ab}$	5.73 (0.53) $^{B\ a}$
		EC	6.59 (0.27) $^{A\ b}$	6.45 (0.29) $^{AB\ b}$	5.54 (0.55) $^{AB\ a}$
Bacteroides	Provinols	AC	7.58 (0.29) $^{C\ c}$	6.80 (0.30) $^{B\ b}$	5.81 (0.35) $^{B\ a}$
		DC	6.75 (0.19) $^{A\ b}$	6.21 (0.10) $^{A\ b}$	4.53 (0.84) $^{A\ a}$
		EC	7.18 (0.16) $^{BC\ b}$	6.69 (0.30) $^{B\ b}$	5.70 (0.62) $^{B\ a}$
	Provinols + *L. plantarum* IFPL93	AC	7.29 (0.28) $^{BC\ c}$	6.54 (0.40) $^{AB\ b}$	5.68 (0.05) $^{B\ a}$
		DC	7.09 (0.35) $^{AB\ b}$	6.14 (0.26) $^{A\ a}$	5.48 (0.87) $^{B\ a}$
		EC	7.12 (0.11) $^{B\ b}$	6.71 (0.35) $^{B\ b}$	5.57 (0.47) $^{B\ a}$
Prevotella	Provinols	AC	7.40 (0.33) $^{C\ c}$	5.67 (0.21) $^{C\ b}$	4.21 (0.13) $^{A\ a}$
		DC	5.61 (0.46) $^{A\ b}$	3.93 (0.14) $^{A\ a}$	3.40 (1.61) $^{A\ a}$
		EC	6.40 (0.60) $^{B\ c}$	5.04 (0.17) $^{B\ b}$	4.30 (0.47) $^{A\ a}$
	Provinols + *L. plantarum* IFPL93	AC	7.56 (0.52) $^{C\ c}$	5.24 (0.40) $^{BC\ b}$	3.94 (0.10) $^{A\ a}$
		DC	6.24 (0.73) $^{AB\ b}$	4.32 (0.47) $^{A\ a}$	3.66 (0.62) $^{A\ a}$
		EC	6.25 (0.42) $^{AB\ c}$	5.11 (0.29) $^{B\ b}$	4.18 (0.61) $^{A\ a}$
Enterobacteriaceae	Provinols	AC	8.40 (0.41) $^{C\ c}$	7.42 (0.20) $^{BC\ b}$	6.29 (0.60) $^{AB\ a}$
		DC	7.22 (0.62) $^{A\ b}$	6.28 (0.32) $^{A\ ab}$	5.10 (1.31) $^{A\ a}$
		EC	8.22 (0.45) $^{BC\ b}$	7.49 (0.66) $^{BC\ b}$	6.48 (0.64) $^{B\ a}$
	Provinols + *L. plantarum* IFPL93	AC	8.15 (0.70) $^{BC\ b}$	6.91 (0.35) $^{AB\ a}$	6.15 (0.40) $^{AB\ a}$
		DC	7.46 (0.58) $^{AB\ b}$	6.67 (0.94) $^{AB\ ab}$	5.70 (0.97) $^{AB\ a}$
		EC	8.43 (0.66) $^{C\ b}$	7.94 (0.29) $^{C\ b}$	6.29 (0.66) $^{AB\ a}$
Blautia coccoides-Eubacterium rectale group	Provinols	AC	7.08 (0.27) $^{B\ c}$	6.65 (0.13) $^{B\ b}$	5.53 (0.29) $^{B\ a}$
		DC	6.36 (0.53) $^{A\ b}$	5.82 (0.30) $^{A\ b}$	4.20 (0.90) $^{A\ a}$
		EC	7.05 (0.14) $^{B\ b}$	6.73 (0.25) $^{B\ b}$	5.28 (1.07) $^{B\ a}$
	Provinols + *L. plantarum* IFPL93	AC	6.75 (0.41) $^{AB\ b}$	6.33 (0.26) $^{B\ b}$	5.31 (0.08) $^{B\ a}$
		DC	6.74 (0.64) $^{AB\ b}$	5.76 (0.45) $^{A\ a}$	5.05 (0.63) $^{AB\ a}$
		EC	6.97 (0.21) $^{B\ b}$	6.75 (0.42) $^{B\ b}$	5.43 (0.71) $^{B\ a}$
Clostridium leptum	Provinols	AC	4.80 (0.71) $^{A\ b}$	4.77 (0.14) $^{A\ b}$	3.81 (0.31) $^{A\ a}$
		DC	5.49 (0.56) $^{B\ b}$	4.95 (0.38) $^{A\ b}$	3.53 (0.53) $^{A\ a}$
		EC	6.35 (0.18) $^{D\ b}$	5.90 (0.31) $^{B\ b}$	4.73 (0.91) $^{B\ a}$
	Provinols + *L. plantarum* IFPL93	AC	4.49 (0.30) $^{A\ b}$	4.44 (0.27) $^{A\ b}$	3.68 (0.06) $^{A\ a}$
		DC	5.77 (0.61) $^{BC\ b}$	4.90 (0.54) $^{A\ ab}$	4.34 (0.53) $^{AB\ a}$
		EC	6.31 (0.18) $^{CD\ b}$	6.00 (0.42) $^{B\ b}$	4.75 (0.74) $^{B\ a}$
Ruminococcus	Provinols	AC	3.01 (0.19) $^{A\ c}$	2.44 (0.14) $^{A\ b}$	1.33 (0.20) $^{A\ a}$
		DC	4.56 (0.62) $^{B\ b}$	4.14 (0.32) $^{B\ b}$	2.50 (2.55) $^{B\ a}$
		EC	5.54 (0.13) $^{D\ b}$	5.20 (0.34) $^{C\ b}$	3.90 (1.01) $^{C\ a}$
	Provinols + *L. plantarum* IFPL93	AC	2.77 (0.22) $^{A\ c}$	2.23 (0.35) $^{A\ b}$	1.44 (0.14) $^{A\ a}$
		DC	4.91 (0.69) $^{BC\ b}$	4.11 (0.65) $^{B\ ab}$	3.44 (0.50) $^{C\ a}$
		EC	5.37 (0.20) $^{CD\ b}$	5.39 (0.58) $^{C\ b}$	3.95 (0.82) $^{C\ a}$
Butyryl-CoA:acetate-CoA transferase gene	Provinols	AC	6.33 (0.04) $^{A\ c}$	5.91 (0.17) $^{A\ b}$	5.42 (0.11) $^{A\ a}$
		DC	6.27 (0.37) $^{A\ b}$	5.89 (0.27) $^{A\ ab}$	5.50 (0.43) $^{A\ a}$
		EC	7.01 (0.35) $^{C\ b}$	6.40 (0.25) $^{B\ a}$	5.96 (0.31) $^{B\ a}$
	Provinols + *L. plantarum* IFPL93	AC	6.28 (0.27) $^{A\ c}$	5.83 (0.20) $^{A\ b}$	5.51 (0.07) $^{A\ a}$
		DC	6.46 (0.52) $^{AB\ b}$	5.85 (0.39) $^{A\ ab}$	5.55 (0.24) $^{AB\ a}$
		EC	6.91 (0.22) $^{BC\ b}$	6.53 (0.18) $^{B\ b}$	5.76 (0.31) $^{AB\ a}$

Data are expressed as means and standard deviation (SD). For a given microbial group analyzed, different capital letters denote significant differences ($P < 0.05$) between compartments in the presence/absence of *L. plantarum* IFPL935. For the same row, different lowercase letters denote significant differences ($P< 0.05$) along the incubation time (for a given compartment)

IV.4.2 Fermentative and proteolytic activities

An important metabolic activity of the gut microbiota is the formation of short-chain fatty acids (SCFAs). Initial SCFAs content was higher in the batches containing human intestinal microbiota from the DC and EC vessels (Fig. IV.1a). During incubation, the higher increase in SCFAs production was measured in the AC vessels although SCFAs increased in all batches indicating that microbiota remained metabolically active. The increase in SCFAs production was attributed mainly to an increase in acetate(r= 0.95), propionate (r=0.86) and butyrate(r=0.23). In general, acetate content was lower in the batches incubated with *L. plantarum* IFPL935 and this was found to be significant in the DC and EC batches (data not shown). This remark correlates with the significantly lower levels of SCFAs found in the DC and EC batches (48 h) when *L. plantarum* IFPL935 was added (Fig. IV.1a). In contrast, and although the differences were not significant, a higher butyrate production (up to 29%) was found in all the batches incubated with *L. plantarum* IFPL935. Also, the higher increase in the butyrate production was detected in the AC batches (Fig. IV.1b).

Ammonium concentration, a marker for proteolytic activity, was higher in the batches inoculated with the DC and EC microbiota (average 20.62 ± 1.12 mmol/L) than in the AC batches (average 12.14 ± 2.21 mmol/L) at the onset of the incubation. However, a marked increase in ammonium production was noticed in the course of the incubation (48 h) in the batches containing the AC microbiota in contrast to that of DC and EC batches, where the figures remained stable (data not shown). Furthermore, ammonium concentration was not found to be significantly different in the batches incubated with IFPL935, except for EC batches (24 h) where this concentration was lower ($P < 0.05$) in batches with IFPL935 (16.08 ± 1.49 mmol/L) than in the control ones (21.55 ± 0.43 mmol/L) (results not shown).

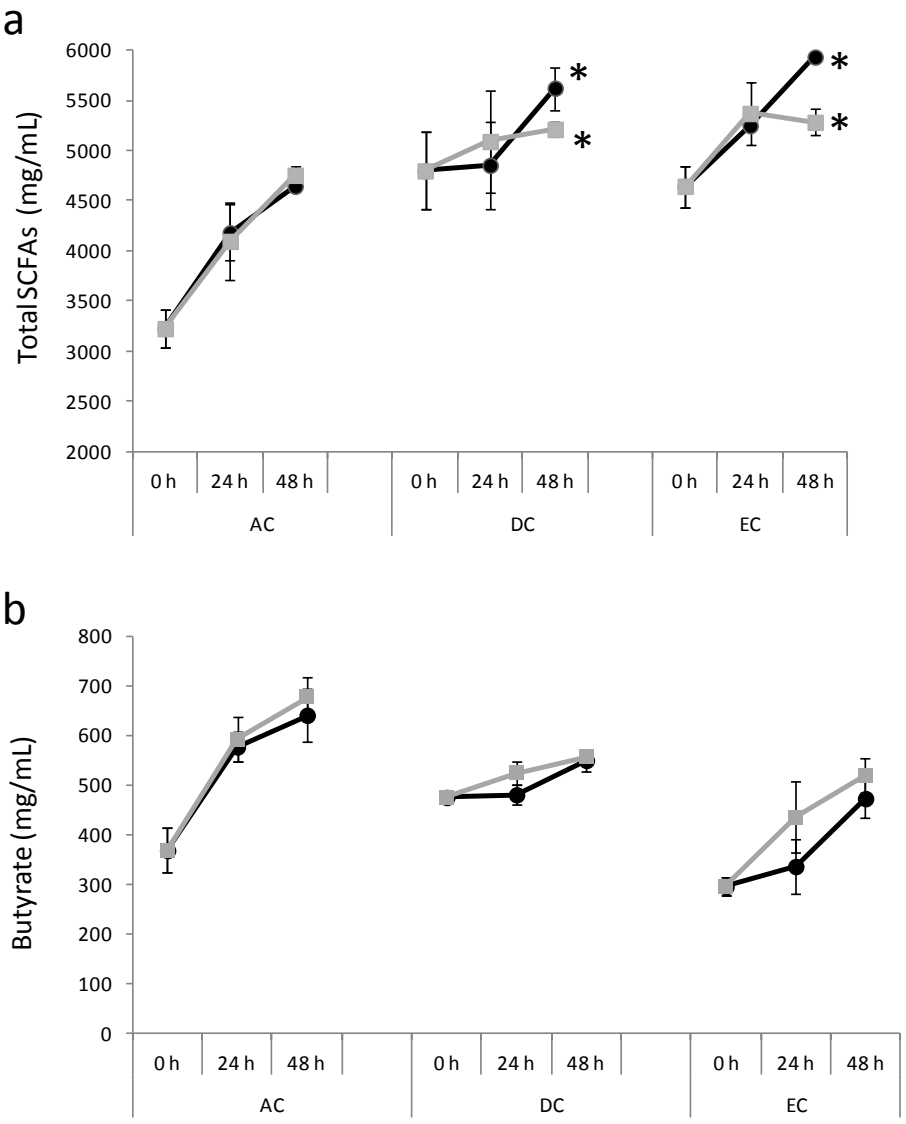

Figure IV.1. Average total SCFAs (a) and butyrate (b) production during the incubation of the microbiota representing the different colon compartments (ascendens, AC; descendens DC; effluent, EC) with Provinols (black circles) and Provinols plus *L. plantarum* IFPL935 (gray squares). For the batches containing microbiota for the same colon compartment, an asterisk indicates significant differences when *L. plantarum* IFPL935 was added.

IV.4.3 Microbial metabolism of phenolic compounds

A total of 28 microbial-derived phenolic metabolites, including benzoic acids, phenols, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones and cinnamic acids, were found when incubating the commercial wine extract with the microbiota from the three different colonic compartments (AC, DC and EC) (Table IV.3). The total concentration of phenolic metabolites (sum of individual metabolite concentration) increased along the incubation period and turned out to be significantly higher in the DC and EC batches with regard to the AC batches (Fig. IV.2a). Overall, the addition of *L. plantarum* IFPL935 to the colonic microbiota did not have an impact on total concentration of phenolic metabolites, except for the EC batches where the figures were significantly higher when IFPL935 was added (24h). In this regard, a three-way ANOVA analysis showed that the main factors that have a significant effect ($P< 0.05$) on the total concentration of phenolic metabolites were the incubation time and the colon region-specific microbiota (AC, DC and EC) (Table IV.3).

When analyzing specific phenolic metabolites, the highest concentrations were found for phenylacetic and 4-hydroxy-5-(phenyl)-valeric acids (21 and 119 µg/mL, respectively), followed by other phenylacetic (3,4-dihydroxy- and 4-hydroxy-) and phenylpropionic (3,4-dihydroxy- and 4-hydroxy-) acids (<8 µg/mL) (Table IV.3). Furthermore, addition of *L. plantarum* IFPL935 resulted to have a significant effect on the 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (i.e. diphenylpropanol) and 4-hydroxy-5-(3'-hydroxyphenyl)-valeric acid concentrations measured. Thus, as depicted in Fig. IV.2b, addition of *L. plantarum* IFPL935 significantly increased the concentration of the initial microbial ring-fission catabolite of catechins and procyanidins, diphenylpropanol, in the batches containing DC and EC microbiota (6 h) and in those harboring AC microbiota (48 h). Similarly, the production of

4-hydroxy-5-(3’-hydroxyphenyl)-valeric acid, an intermediate metabolite derived from 4-hydroxy-5-(3’,4'-dihydroxyphenyl)-valeric acid which in turn comes out from the diphenylpropanol (Monagas et al., 2010), followed the same trend (results not shown). Moreover, (-)-epicatechin, one of the main flavan-3-ols present in the wine extract, was completely degraded by the DC and EC microbiota at 24 h of incubation, while degradation of (-)-epicatechin was only noticeable at 48 h in the AC batches, being greater in the presence of *L. plantarum* IFPL935 (Fig. IV.2c). Similar trends were observed for other compounds present in the wine extract such as (+)-catechin and procyanidin dimers and trimers (results not shown).

Table IV.3. Concentration of the Phenolic Metabolites Measured[a] and Main Effects of the Factors[b]

				Factors Effects		
	Mean (µg/mL)	Minimum (µg/mL)	Maximum (µg/mL)	Time	Microbiota	*L. plantarum* IFPL935
	Benzoic acids					
Gallic acid	0.85 (0.53)	0.00	2.27		*	
Protocatechuic acid	0.60 (0.21)	0.23	1.07	*	*	
4-Hydroxybenzoic acid	0.23 (0.04)	0.15	0.33	*	*	
Vanillic acid	0.16 (0.08)	0.04	0.33	*	*	
Syringic acid	0.32 (0.15)	0.04	0.72	*	*	
Benzoic acid	0.39 (0.08)	0.23	0.61	*	*	
Salicylic acid	0.16 (0.05)	0.09	0.30	*	*	
	Phenols					
Phloroglucinol	0.04 (0.10)	0.00	0.60	*		
Pyrogallol	0.08 (0.20)	0.00	0.90	*	*	
Catechol/Pyrocatechol	0.13 (0.11)	0.00	0.39	*	*	
	Phenylacetic acids					
3,4-Dihydroxyphenylacetic acid	0.43 (0.67)	0.00	5.01	*		
4-Hydroxyphenylacetic acid	4.18 (1.73)	0.63	7.79		*	
3-Hydroxyphenylacetic acid	0.09 (0.05)	0.00	0.19	*	*	
4-Hydroxy-3-methoxyphenylacetic acid	0.01(0.02)	0.00	0.06	*	*	
Phenylacetic acid	14.66 (4.11)	5.10	21.08	*	*	
	Phenylpropionic acids					
3-(3,4-Dihydroxyphenyl)-propionic acid	1.97 (1.52)	0.00	5.22	*	*	
3-(4-Hydroxyphenyl)-propionic acid	2.34 (1.45)	0.22	5.33	*	*	
3-(3-Hydroxyphenyl)-propionic acid	0.20 (0.54)	0.00	2.75	*	*	
Phenylpropionic acid	0.66 (0.39)	0.00	1.47	*	*	
	Valeric acids					
4-Hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid	0.50 (1.06)	0.00	4.08	*		
4-Hydroxy-5-(3'-hydroxyphenyl)-valeric acid	0.06 (0.19)	0.00	1.04	*		*
4-Hydroxy-5-(phenyl)-valeric acid	17.03 (34.31)	0.00	119.27	*	*	
	Valerolactones					
5-(3',4'-dihydroxyphenyl)-γ-valerolactone	0.45 (0.89)	0.00	3.37	*		
	Cinnamic acids					
Caffeic acid	0.27 (0.43)	0.00	1.29	*		
p-Coumaric acid	0.16 (0.23)	0.00	0.78	*		
Ferulic acid	0.02 (0.03)	0.00	0.11	*		
Isoferulic acid	0.02 (0.03)	0.00	0.11	*	*	
	Other metabolites					
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol	0.07 (0.19)	0.00	0.99	*	*	*
∑ Phenolic metabolites	43.90 (39.33)	7.52	153.95	*	*	

[a]Mean, standard deviation (SD), and range of variation (minimum and maximum) of the phenolic metabolites concentration measured after incubation of the wine extract with the different colon region microbiota. [b]Statistical significance (*) ($P < 0.05$) of the main effects of the factors: time (0, 6, 24, and 48 h), microbiota (AC, DC and EC), and addition of *L. plantarum* IFPL935 is also shown

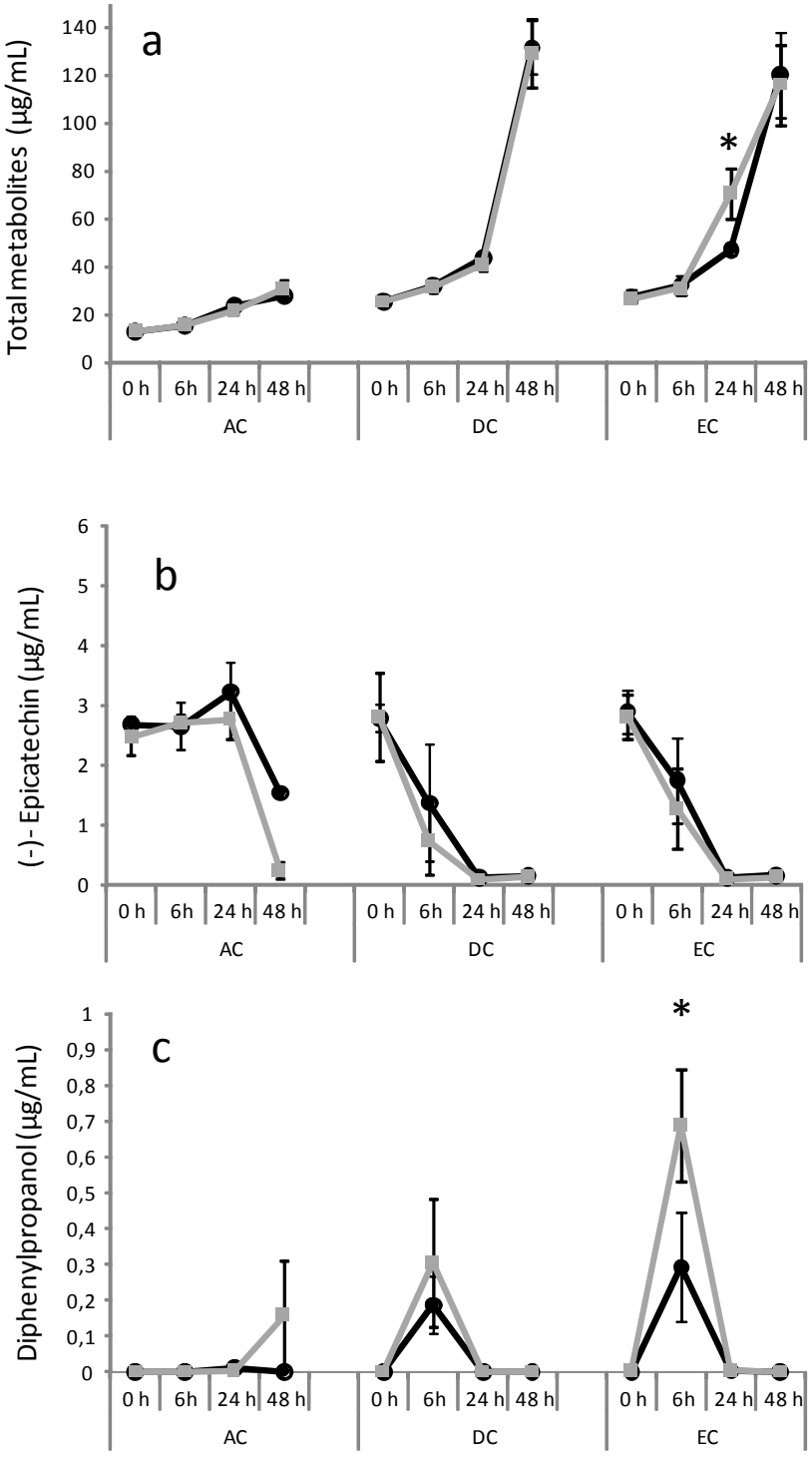

Figure IV.2. Production of total microbial-derived phenolic metabolites (a) and 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (b) and the disappearance of (−)-epicatechin (c) during the incubation of the wine extract with the three different colon region microbiota (AC, DC, and EC) with Provinols (black circles) and Provinols plus *L. plantarum* IFPL935 (gray squares). For the batches containing microbiota for the same colon compartment, an asterisk denotes significant differences with regard to the batches where *L. plantarum* IFPL935 was added

Principal Component Analysis (PCA) was performed to obtain a simplified view of the changes in phenolic microbial metabolism under the different conditions. Two principal components, PC1 and PC2, which explained 49% of the total variance, were found. For a better understanding of the data, mean values of the scores of the triplicate assays in the different time periods (0, 6, 24 and 48 h), considering the distinct colon region-specific microbiota and the addition of *L. plantarum* IFPL935, were plotted in the plane delimited by the first two principal components (Figure IV.3). PC1, which explained 36.1% of the variance, reflected overall changes occurring during the time-course of the microbial catabolism of the wine polyphenols. This component was negatively correlated (loadings > 0.72) with 3-(4-dihydroxyphenyl)-propionic acid (-0.85), phenylpropionic acid (-0.85), salicylic acid (-0.82), protocatechuic acid (-0.79), 3-(3,4-dihydroxyphenyl)-propionic acid (-0.79), catechol/pyrocatechol (-0.76) and phenylacetic acid (-0.73). Therefore, lower values of PC1 corresponded to higher concentrations of the compounds, which were measured at longer incubation times, mainly in the vessels containing the DC and EC microbiota. Interestingly, PC2, which explained 12.9% of the variance, showed differences in the phenolic metabolic profile of the batches containing the same colon region-specific microbiota when *L. plantarum* IFPL935 was added. These differences in the metabolites profiles were detected at 6 h for DC and EC batches, and for the AC batches at 24-48 h of incubation. In all cases, higher values of PC2 were related to the addition of IFPL935.

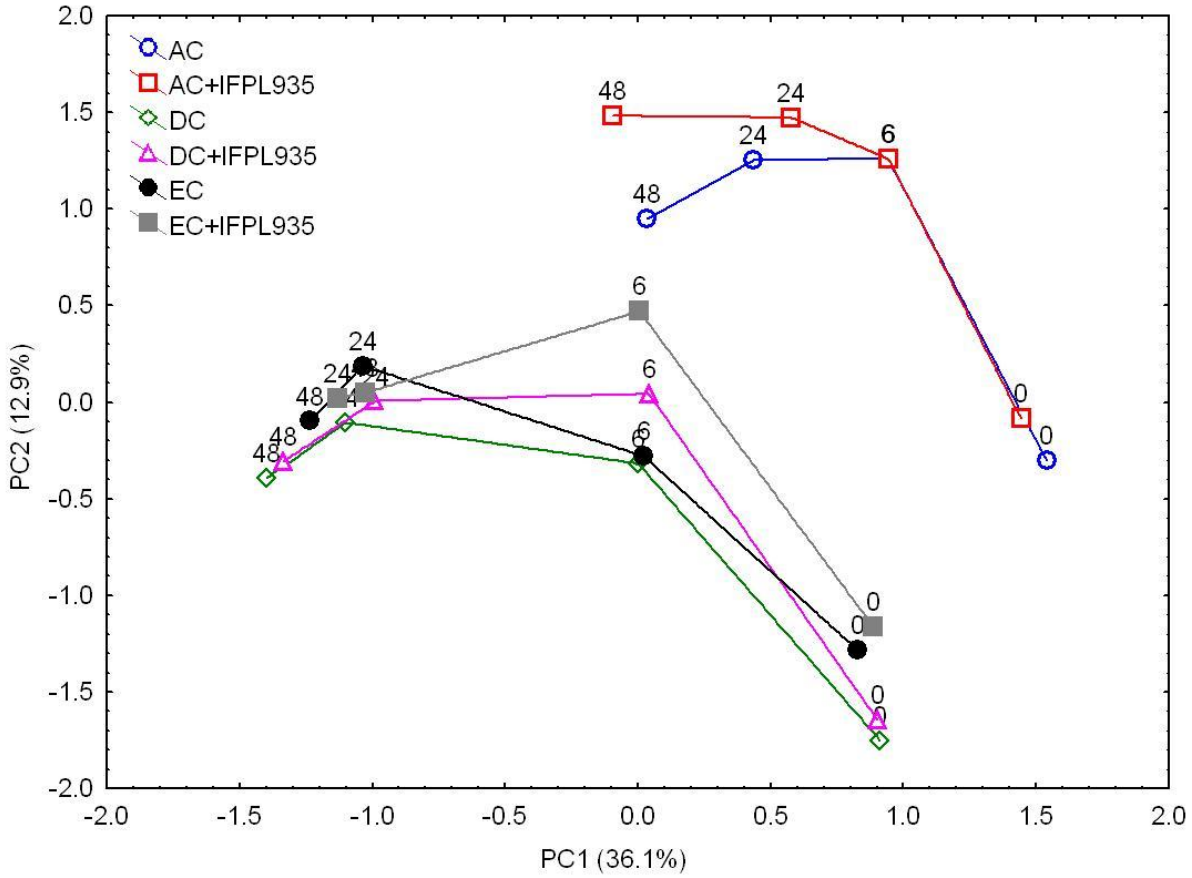

Figure IV.3. Representation of the samples in the plane defined by the first two principal components (PC1 and PC2) resulted from a PCA of microbial-derived phenolic metabolites for the different colonregion-specific microbiota (AC, DC, and EC) and considering the addition of *L. plantarum* IFPL935 at different incubation times (0, 6, 24, and 48 h).

IV.5 DISCUSSION

Bearing in mind that the composition and metabolic activity of the intestinal microbiota is colon-region dependent, this study was aimed to evaluate the contribution of *L. plantarum* IFPL935 to the colonic metabolism of wine polyphenols using *in vitro* batch fermentations inoculated with human microbiota developed in different colonic compartments of the SHIME. The SHIME has been reported to be able to simulate reproducible and highly diverse microbial communities which are colon region specific (Van den Abbeele et al., 2010).

The colonic microbiota plays an important role in the microbial catabolism of dietary polyphenols. It has been estimated that 90-95% of dietary polyphenols are not absorbed in the small intestine and therefore accumulate in the colon (Clifford et al., 2004). Therefore, the bioactivity of these compounds is largely dependent on the microbiota activity. In this study, a wide range of potential phenolic metabolites arising from flavan-3-ols catabolism, including first and intermediate metabolites (diphenylpropanol, 4-hydroxy-5-(3'-hydroxyphenyl)-valeric acid) and end products (hydroxypropionic acid, hydroxyacetic acids, cinnamic acids) were targeted during the course of the batch fermentations. Besides, other metabolites detected such as O-methylated benzoic acids including syringic and vanillic acids, could also arise from the catabolism of anthocyanins and other flavonoids also present in the red wine extract, as it has been previously reported (Sánchez-Patán et al., 2012). Furthermore, the microbial metabolic activity on the red wine extract polyphenols appears to vary according the specific colon region microbiota as different phenolic metabolite profiles were detected in the distinct batch fermentations. Thus, production of phenolic intermediate metabolites and phenolic acids were detected earlier during the incubation and reached higher concentrations in the batches containing human intestinal microbiota simulating the distal colon regions (DC and

EC) when compared to the batches inoculated with microbiota from the ascending colon region (AC) (Fig. IV.2), suggesting that bacterial conversion of wine polyphenols was more favorable in the distal colon. This colon site specificity of the microbial transformations is in agreement with previous studies where a higher microbial conversion of phenolic compounds was found in the distal compartments (Possemiers et al., 2006; Van Dorsten et al., 2012).

Microbial catabolism of polyphenols comprises a series of chain reactions, leading to numerous intermediate metabolites and end products (Aura et al., 2008; Requena et al., 2010; Monagas et al., 2010). The catabolism of flavan-3-ols starts with the reductive cleavage of the heterocyclic C ring, resulting in the formation of diphenylpropan-2-ol, followed by the breakdown of the A ring and further lactonization into phenylvalerolactones derivatives, specifically into 5-(3,4-dihydroxyphenyl)-γ-valerolactone. However, knowledge about intestinal bacteria involved in the cleavage of the C-ring conversion is limited to only a few strains isolated from human feces such as *Eubacterium* sp. strain SDG-2 and *Eggerthella lenta* rK3 (Wang et al., 2011; Kutschera et al., 2011). Previous findings showed that besides other polyphenol metabolic activities, *L. plantarum* IFPL935 was capable in pure culture of cleaving the heterocyclic ring of monomeric flavan-3-ols, giving rise to the first metabolite of the microbial catabolic pathway (Tabasco et al., 2011; Sánchez-Patán et al., 2012). Hence, the results here presented demonstrate the capability of *L. plantarum* IFPL935 of initiating wine polyphenol catabolism in the complex environment of colonic microbiota, as seen for the quicker disappearance of flavan-3-ols (i.e., (-)-epicatechin), and production of diphenylpropanol and other intermediate metabolites immediately-derived from it in the batches incubated with *L. plantarum* IFPL935 (Fig. IV.2). Moreover, changes in the profile of these compounds observed at the first stages of phenolic degradation (after 6 h of incubation in the DC and EC batches, and at 24-48 h of incubation in

the AC batches) (Fig. IV.2) might be associated with the differences observed in microbial communities between the colon region-specific compartments (Table IV.2).

In this regard, the analysis of the microbial communities in the batches representing the AC, DC and EC microbiota further indicate higher variations between *in vitro* batches harboring a different colon region microbiota than those found when *L. plantarum* IFPL935 was added. Thus, AC batches, where the fermentation is very intense with high production of short chain fatty acids (SCFAs), were shown to harbor the major proportion of saccharolytic bacteria (*Bacteroides*, *Bifidobacterium*, *Prevotella*). Therefore, most of the oligosaccharides used as prebiotics in the functional food industry are predominantly fermented in the proximal colon (Roberfroid et al., 2010). By contrast, the microbial community, represented by *Clostridium* groups was found in major numbers in the batches inoculated with microbiota simulating the distal regions (DC and EC vessels) where the putrefactive processes become quantitatively more important, although proteolysis occurs along the entire colon.

Although knowledge about specific gut bacteria capable to degrade wine polyphenols such as flavan-3-ols is still scarce, it may be difficult to identify a single bacterium capable of exhibiting the whole catabolic pathway, but rather the catabolism may be carried out by different bacteria species acting at some steps of the different degradation pathways. The formation of diphenylpropanol and other intermediate metabolites and its further transformation into phenylpropionic, phenylacetic, and benzoic acids could be critical steps delimiting the rate and extent of red wine polyphenols catabolism, thus influencing bioavailability and bioactivity of these compounds *in vivo*. However, total production of phenolic metabolites seemed not to be affected by the presence of *L. plantarum* IFPL935 at further degradation steps or longer

incubation times (Fig. IV.2a), suggesting that the colonic bacteria that undergo further flavan-3-ol degradation exhibit limited catabolism capability. This limited bacterial activity in phenolic metabolism at further degradation steps, could be explained, at least partly, because of the decrease in nutrient concentration that occurs in batch culture model as incubation time progresses. Further studies will be performed using gastrointestinal dynamic models where microbial populations and microbe-mediated metabolic effects can be dynamically monitored in the different colon regions and along the incubation time avoiding the limitation of nutrients and phenolic substrates.

Besides polyphenol catabolism, special attention was given to the butyrate-producing bacteria as they play an important role in the human colon, supplying energy to the gut epithelium and regulating host cell responses (Macfarlane and Macfarlane 2012). Butyrate-producing bacteria represent a functional group rather than a phylogenetic group and, as their distribution within bacterial clusters is uneven. A molecular approach based on the enumeration of the butyryl-CoA:acetate CoA transferase gene (BCoAT) was used for estimating the number of butyrate-producing bacteria in samples containing a complex microbiota (Louis et al., 2010). BCoAT gene copy numbers significantly decreased along the incubation (48 h) in all batches representing the distinct colon region-specific microbiota, in agreement with the decrease in bacterial population numbers along the incubation (Table IV.2). More meaningfully, data analysis showed a significant correlation ($P< 0.05$) between counts of this functional gene and the presence of *C. leptum*, *B. coccoides-E. rectale* group and to a lesser extent to *Ruminococcus*, confirming previous studies in which bacteria belonging to *Clostridium* (Cluster IV and XIVa) were pointed out as important butyrate producers playing a key role in butyric acid production (Louis et al., 2010).

Despite the decrease of butyrate producers, a higher butyrate production was detected in the batches containing the ascending colon microbiota (AC) at the end of the incubation compared to DC and EC batches (Fig. IV.1b). This is in agreement with the fact that most of the fermentative metabolism takes place in the AC but the results also showed that the presence of *L. plantarum* IFPL935 tended to increase butyrate production while in turn decrease acetate and in general total SCFAs concentration (Fig. IV.1a). In this regard, when analyzing the SCFAs production, cross-feeding interactions between colon bacteria should be taken into account. On one hand, bifidobacteria and lactobacilli strains are potentially able to produce lactate that can be further turned into butyrate and propionate through cross-feeding by other bacteria such as *Eubacterium hallii* within *B. coccoides* cluster (Belenguer et al., 2006). This could explain the favorable effect of the presence of *L. plantarum* 935 on butyric acid production. On the other hand, bacteria of the *B. coccoides* cluster can also convert acetate produced by the main acetate producers such as *Bacteroides* and *Prevotella* (Macfarlane and Macfarlane, 2012) into butyric acid (Belenguer et al., 2006; Barcenilla et al., 2000).

In summary, the results highlighted confirm the capability of *L. plantarum* IFPL935 to initiate flavan-3-ols catabolism when added to a complex human intestinal microbiota, which may have an impact on the bioavailability of these dietary polyphenols and therefore on its biological *in vivo* effects. Besides, *L. plantarum* IFPL935 may have an impact on SCFAs production, and in particular butyrate, which is known to play an important role for maintenance of gut health.

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IV.6 REFERENCES

Alander, M.; De Smet, I.; Nollet, L.; Verstraete, W.; Von Wright, A.; Mattila-Sandholm, T. The effect of probiotic strains on the microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). Int. J. Food Microbiol. 1999, 46, 71-79.

Aura, A.M. Microbial metabolism of dietary phenolic compounds in the colon. Phytochem. Rev. 2008, 7, 407–429.

Barcenilla, A.; Pryde, S.E.; Martin, J.C.; Duncan, S.H.; Stewart, C.S.; Henderson, C.; Flint, H.J. Phylogenetic relationships of butyrate-producing bacteria from the human gut. Appl. Environ. Microbiol. 2000, 66, 1654-1661.

Bartosch, S.; Fite, A.; Macfarlane, G.T.; McMurdo, M.E.. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 2004, 70, 3575-3581.

Belenguer, A.; Duncan, S.H.; Calder, G.; Holtrop, G.; Louis, P.; Lobley, G.E.; Flint, H.J. Two routes of metabolic cross-feeding between bifidobacteria and butyrate-producing anaerobes from the human gut. Appl Environ Microbiol. 2006, 72, 3593–3599.

Bremner, J.M.; Keeney, R.D. Steam distillation methods for determination of ammonium, nitrate and nitrite. Anal. Chem. Acta 1965, 32, 485–495.

Byun, R.; Nadkarni, M.A.; Chhour, K.L.; Martin, F.E.; Jacques, N.A.; Hunter, N. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. J. Clin. Microbiol. 2004, 42, 3128-3136.

Clifford, M.N. Diet-derived phenols in plasma and tissues and their implications for health. Planta Med. 2004, 70, 1103-1114.

Crozier, A. Dietary phenolics, absorption, mammalian and microbial metabolism and colonic health. Mol. Nutr. Food Res. 2009, 53, S5-6

Dolara, P.; Luceri, C.; De, F. C.; Femia, A. P.; Giovannelli, L.; Caderni, G.; Cecchini, C.; Silvi, S.; Orpianesi, C.; Cresci, A. Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutat. Res. 2005, 591, 237–246.

Fontecha, J.; Peláez, C.; Juárez, M.; Requena, T.; Gómez, C.; Ramos, M. Biochemical and microbiological characteristics of artisanal hard goat's cheese. J. Dairy Sci. 1990, 73, 1150−1157.

Gibson, G.R.; Fuller, R. Aspects of *in vitro* and *in vivo* research approaches directed toward identifying probiotics and prebiotics for human use. J. Nutr. 2000, 130, 391S-395S.

Godornes, C.; Leader, B.T.; Molini, B.J.; Centurion-Lara, A.; Lukehart, S.A. Quantification of rabbit cytokine mRNA by real-time RT-PCR. Cytokine 2007, 38, 1-7.

Griffiths, R.I.; Whiteley, A.S.; O'Donnell, A.G.; Bailey, M.J.. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNAbased microbial community composition. Appl. Environ. Microbiol. 2000, 66, 5488–5491.

Hackman, R.M.; Polagruto, J.A.; Yan Zhu, Q.; Sun, B.; Fujii, H.; Keen, C.L. Flavanols: digestion, absorption and bioactivity. Phytochem. Rev. 2008, 7, 195–208

Jiménez-Girón, A; Queipo-Ortuño, M.I.; Boto-Ordóñez, M.; Muñoz-González, I.; Sánchez-Patán, F.; Monagas, M.; Martín-Álvarez, P.J.;

Murri, M.; Tinahones, F.J.; Andrés-Lacueva, C.; Bartolomé, B.; Moreno-Arribas, M.V. Comparative study of microbial-derived phenolic metabolites in human feces after intake of gin, red wine, and dealcoholized red wine. J. Agric. Food Chem. 2013, 61, 3909–3915.

Kowalchuk, G.A.; Bodelier, P.L.E.; Heilig, G.H.J.; Stephen, J.R.; Laanbroek, H.J. Community analysis of ammoniaoxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. 1998, 27, 339–350.

Kutschera, M.; Engst, W.; Blaut, M.; Braune, A. Isolation of catechin-converting human intestinal bacteria. J. Appl. Microbiol. 2011, 111, 165−175

Leser, T.D.; Amenuvor, J.Z.; Jensen, T.K.; Lindecrona, R.H.; Boye, M. and Moller, K. Culture independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl. Environ. Microbiol. 2002, 68, 673-690.

Louis, P.; Flint, H.J. Development of a semiquantitative degenerate real-time PCR-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. Appl. Environ. Microbiol. 2007, 73, 2009-2012.

Louis, P.; Young, P.; Holtrop, G.; Flint, H.J. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ. Microbiol. 2010, 12, 304-314.

Macfarlane, G.T.; Macfarlane, S. Bacteria, colonic fermentation, and gastrointestinal health. J. AOAC Int. 2012, 95, 50-60.

Matsuki, T.; Watanabe, K.; Fujimoto, J.; Miyamoto, Y.; Takada, T.; Matsumoto, K.; Oyaizu, H.; Tanaka, R. Development of 16S rRNA-gene-

targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 2002, 68, 5445–5451.

Matsuki, T.; Watanabe, K.; Fujimoto, J.; Takada, T., Tanaka, R. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl. Environ. Microbiol. 2004, 70, 7220-7228.

Molly, K.; van de Woestijne, M.; Verstraete, W. Development of a 5-step multichamber reactor as a simulation of the human intestinal microbial ecosystem. Appl. Microbiol. Biotechnol. 1993, 39, 254–258.

Molly, K.; van de Woestyne, M.; DeSmet, I.; Verstraete, W. Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism associated activities. Microb. Ecol. Health Dis. 1994, 7, 191–200.

Monagas, M.; Urpi-Sarda, M.; Sanchez-Patan, F.; Llorach, R.; Garrido, I.; Gomez-Cordoves, C.; Andres-Lacueva, C.; Bartolome, B. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food Funct. 2010, 1, 233-253.

Nübel, U.; Engelen, B.; Felske, A.; Snaidr, J.; Wieshuber, A.; Amann, R. I.; Ludwig, W.; Backhaus, H. Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J. Bacteriol. 1996, 178, 5636–5643.

Possemiers, S.; Bolca, S.; Grootaert, C.; Heyerick, A.; Decroos, K.; Dhooge, W.; De Keukeleire, D.; Rabot, S.; Verstraete, W.; Van de Wiele, T. 2006. The prenylflavonoid isoxanthohumol from hops (Humulus lupulus

L.) is activated into the potent phytoestrogen 8-prenylnaringenin *in vitro* and in the human intestine. J. Nutr. 2006, 136, 1862–1867.

Possemiers, S.; Bolca, S.; Verstraete, W.; Heyerick, A. The intestinal microbiome: a separate organ inside the body with the metabolic potential to influence the bioactivity of botanicals. Fitoterapia. 2011, 82, 53-66.

Queipo-Ortuño, M.I.; Boto-Ordoñez, M.; Murri, M.; Gomez-Zumaquero, J.M.; Clemente-Postigo, M.; Estruch, R.; Cardona Diaz, F.; Andrés-Lacueva, C.; Tinahones, F.J. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. Am. J. Clin. Nutr. 2012, 95, 1323-1334.

Ramirez-Farias, C.; Slezak, K.; Fuller, Z.; Duncan, A.; Holtrop, G.; Louis P. Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. Br. J. Nutr. 2009, 101, 541-550

Requena, T.; Requena, T.; Monagas, M.; Pozo-Bayon, M.A.; Martin-Alvarez, P.J.; Bartolome, B.; del Campo, R.; Avila, M.; Martinez-Cuesta, M.C.; Pelaez, C.; Moreno-Arribas, M.V. Perspectives of the potential implications of wine polyphenols on human oral and gut microbiota. Trends Food Sci. Technol. 2010, 21, 332-344.

Rinttilä, T.; Kassinen, A.; Malinen, E.; Krogius, L.; Palva, A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J. Appl. Microbiol. 2004, 97, 1166-1177.

Del Rio, D.; Rodriguez-Mateos, A.; Spencer, J.P.E.; Tognolini, M.; Borges, G.; Crozier, A. Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid. Redox Signal. 2013, 18, 1818-1892.

Roberfroid, M.; Gibson, G.R.; Hoyles, L.; McCartney, A.L.; Rastall, R.; Rowland, I.; Wolvers, D.; Watzl, B.; Szajewska, H.; Stahl, B.; Guarner, F.; Respondek, F.; Whelan, K.; Coxam, V.; Davicco, M.J.; Léotoing, L.; Wittrant, Y.; Delzenne, N.M.; Cani, P.D.; Neyrinck, A.M.; Meheust, A. Prebiotic effects: metabolic and health benefits. Br. J. Nutr. 2010, 104 Suppl 2:S1-63.

Sánchez-Patán, F.; Cueva, C.; Monagas, M.; Walton, G.E.; Gibson, G. R.; Quintanilla-López, J. E.; Lebrón-Aguilar, R.; Martín-Álvarez, P.J.; Moreno-Arribas, M. V.; Bartolomé, B. *In vitro* fermentation of a red wine extract by human gut microbiota: changes in microbial groups and formation of phenolic metabolites. J. Agric. Food Chem. 2012, 60, 2136-2147.

Sánchez-Patán, F.; Monagas, M.; Moreno-Arribas, M. V.; Bartolomé, B.. Determination of microbial phenolic acids in human faeces by UPLC-ESI-TQ MS J. Agric. Food. Chem. 2011, 59, 2241-2247.

Sánchez-Patán, F.; Tabasco, R.; Monagas, M.; Requena, T.; Peláez, C.; Moreno-Arribas, M.V.; Bartolomé, B. Capability of *Lactobacillus plantarum* IFPL935 to catabolize flavan-3-ol compounds and complex phenolic extracts. J. Agric. Food Chem. 2012, 60, 7142-7151.

Selma, M.V.; Espín, J.C.; Tomás-Barberán, F.A. Interaction between phenolics and gut microbiota: role in human health. J. Agric. Food Chem. 2009, 57, 6485-6501.

Sghir, A.; Gramet, G.; Suau, A.; Rochet, V.; Pochart, P.; Dore, J. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. 2000, 66, 2263–2266.

Tabasco, R.; Sánchez-Patán, F.; Monagas, M.; Bartolomé, B.; Moreno-Arribas, M.V.; Peláez, C.; Requena, T. Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth:resistance and metabolism. Food Microbiol. 2011, 28, 1345-1352.

Tzounis, X.; Vulevic, J.; Kuhnle, G. C.; George, T.; Leonczak, J.; Gibson, G. R.; Kwik-Uribe, C.; Spencer, J.P. Flavonol monomer-induced changes to the human faecal microflora. Br. J. Nutr. 2008, 99, 782-792.

Van den Abbeele, P.; Grootaert, C.; Marzorati, M.; Possemiers, S.; Verstraete, W.; Gérard, P.; Rabot, S.; Bruneau, A.; El Aidy, S.; Derrien, M.; Zoetendal, E.; Kleerebezem, M.; Smidt, H.; Van de Wiele, T. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and *Clostridium* cluster IX. Appl. Environ. Microbiol. 2010, 76, 5237–5246.

Van Dorsten, F. A.; Peters, S.; Gross, G.; Gomez-Roldan, V.; Klinkenberg, M.; de Vos, R. C.; Vaughan, E. E.; van Duynhoven, J. P.; Possemiers, S.; van de Wiele, T.; Jacobs, D. M. Gut Microbial Metabolism of Polyphenols from Black Tea and Red Wine/Grape Juice Is Source-Specific and Colon-Region Dependent. J. Agric. Food Chem. 2012, 60, 11331-11342.

van Duynhoven, J.; Vaughan, E.E.; Jacobs, D.M.; Kemperman, R.A.; van Velzen, E.J.; Gross, G.; Roger, L.C.; Possemiers, S.; Smilde, A.K.; Doré, J.; Westerhuis, J.A.; Van de Wiele, T. Metabolic fate of polyphenols in the human superorganism. Proc. Natl. Acad. Sci. USA. 2011, 108, 4531-4538.

Wang, L.Q.; Meselhy, M.R.; Li, Y.; Nakamura, N.; Min, B.S.; Qin, G.W.; Hattori, M.. The heterocyclic ring fission and dehydroxylation of catechins and related compounds by *Eubacterium* sp. strain SDG-2, a human intestinal bacterium. Chem. Pharm. Bull. 2011, 49, 1640-1643.

Waterhouse, A.L. Wine phenolics. Ann. N. Y. Acad. Sci. 2002, 957, 21–36.

V. *LACTOBACILLUS PLANTARUM* IFPL935 IMPACTS COLONIC METABOLISM IN A SIMULATOR OF THE HUMAN GUT MICROBIOTA DURING FEEDING WITH RED WINE POLYPHENOLS

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V.1 ABSTRACT

The colonic microbiota plays an important role in the bioavailibility of dietary polyphenols. This work has evaluated the impact on the gut microbiota of long-term feeding with both a red wine polyphenolic extract and the flavan-3-ol metabolizer strain *Lactobacillus plantarum* IFPL935. The study was conducted in the dynamic Simulator of the Human Intestinal Microbial Ecosystem (SHIME). The feeding of the gut microbiota model with red wine polyphenols caused an initial decrease in the counts of total bacteria in the ascending colon (AC), being *Bacteroides*, *Blautia coccoides*/*Eubacterium rectale* and *Bifidobacterium* the most affected bacterial groups. The bacterial counts recovered to initial numbers faster than the overall microbial fermentation and proteolysis, which seemed to be longer affected by polyphenols. Addition of *L. plantarum* IFPL935 helped to promptly recover total counts, *Lactobacillus* and *Enterobacteriaceae* and led to an increase in lactic acid formation in the AC vessel at the start of the polyphenol treatment as well as butyric acid in the transverse (TC) and descending (DC) vessels after 5 days. Besides, *L. plantarum* IFPL935 favoured the conversion in the DC vessel of monomeric flavan-3-ols and their intermediate metabolites into phenylpropionic acids and in particular 3-(3´-hydroxyphenyl) propionic acid. The results open possibilities of using *L. plantarum* IFPL935 as a food ingredient for helping individuals showing a low polyphenol-fermenting metabotype to increase their colonic microbial capacities of metabolizing dietary polyphenols.

V.2 INTRODUCTION

Moderate consumption of red wine and/or regular consumption of other polyphenol-rich beverages and foods has proved in epidemiological studies to reduce the incidence of certain chronic diseases (Arranz et al. 2012; Kishimoto et al. 2013). Although the health effects of food polyphenols have been repeatedly associated with their free radical scavenging and antioxidant activity, recent evidence has, however, indicated that the effects of antioxidants are less relevant than expected (Tomás-Barberán and Andrés-Lacueva 2012). The bioavailability, absorption and metabolism of polyphenolic compounds are, indeed, key issues required to explain their role in human health. Depending on their chemical structure and food matrix, a high percentage of dietary polyphenols are not absorbed in the small intestine, reaching the colon where they are metabolized by the gut microbiota before being absorbed. Likewise, polyphenols and their resultant metabolites may selectively modulate the gut microbial composition by their antimicrobial or eventually prebiotic-like properties (Queipo-Ortuño et al. 2012; Tzounis et al. 2008). In this sense, an increase in *Lactobacillus* and *Bifidobacterium* species following administration of red wine polyphenols has been reported (Dolara et al. 2005; Queipo-Ortuño et al. 2012). Moreover, a significant correlation of moderate wine consumption with human microbiome composition, but not with enterotype partitioning, has been established (Wu et al., 2011).

Flavonoids are the most abundant polyphenols present in red wine, mainly including flavan-3-ols and anthocyanins (Waterhouse 2002). There are several metabolic pathways proposed for the catabolism of monomeric flavan-3-ol and dimeric procyanidins by the intestinal microbiota (Monagas et al. 2010; Selma et al. 2009). However, bacterial degradation of flavan-3-ols is hindered by the inherent antibacterial effects of these molecules. In previous studies, we have reported that *L.*

plantarum IFPL935 was capable to metabolize galloylated flavan-3-ols leading to the formation of gallic acid, pyrogallol and catechol through galloyl-esterase, decarboxylase and benzyl alcohol dehydrogenase enzyme activities, respectively (Tabasco et al. 2011). The most remarkable feature of *L. plantarum* IFPL935 was its ability to cleave the heterocyclic ring of monomeric flavan-3-ols, giving rise to 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (i.e. diphenylpropanol), which is the first metabolite in the microbial catabolic pathway of flavan-3-ols (Sánchez-Patán et al. 2012b). This activity has only been reported in a few intestinal bacteria to date, such as *Eubacterium* sp. SDG-2, *Eggerthella lenta*, and *Flavonifractor plautii* (Jin and Hattori 2012; Kutschera et al. 2011; Wang et al. 2001). The capability of *L. plantarum* IFPL935 to initiate the catabolism of flavan-3-ols via the formation of diphenylpropanol has also been demonstrated when the strain was incubated in batches with a complex human intestinal microbiota (Barroso et al., 2013). It was also observed that the addition of this strain had an impact on the formation of butyric acid, probably through cross-feeding with colonic butyrate-producing bacteria (Barroso et al., 2013).

To get a deeper insight on the potential use of *L. plantarum* IFPL935 to benefit polyphenols metabolism we have monitored the effect on the gut microbiota upon addition of 1010 cfu of the strain to a daily intake of a polyphenolic extract (equivalent to two 125 ml-glasses of alcohol-free red wine) in a long-term feeding experiment. The study was conducted in the dynamic multireactor gastrointestinal Simulator of the Human Intestinal Microbial Ecosystem (SHIME).

V.3 MATERIALS AND METHODS

V.3.1 Simulator of the Human Intestinal Microbial Ecosystem (Twin-SHIME)

In this study, a Twin-SHIME® (UGent/ProDigest) setup was used by operating two parallel SHIME systems, each one consisting of five consecutive reactors, simulating the stomach, small intestine, ascending colon (AC), transverse colon (TC), and descending colon (DC), as described by Van den Abbeele et al. (2010). At the beginning of the experiment, the AC, TC and DC vessels from the Twin-SHIME systems were all simultaneously inoculated with the same fecal sample from a healthy human volunteer previously identified as a flavan-3-ol metabolizer (unpublished results) to enable comparison of the results between the two parallel experiments. The colonic microbiota was allowed for stabilization reaching the steady state after 3 weeks. The inoculum preparation and the SHIME feed composition during the stabilization period were essentially as described by De Boever et al. (2004). After stabilization of the colonic microbiota, both Twin-SHIME systems were subjected to a 2-week experiment by daily feeding the stomach compartments with 200 mg of a commercial red wine extract, ProvinolsTM (Safic-Alcan Especialidades, Barcelona, Spain), which corresponds to a daily polyphenol intake of two small glasses of red wine (250 ml). The total phenolic content of the red wine extract was 474 mg of gallic acid equivalents per g (2.79 mmol/g) and its composition was reported before (Sánchez-Patán et al. 2012a), being flavan-3-ols the main phenolic compounds. In addition, one of the Twin-SHIME systems was simultaneous fed with a daily dose of 1010 cfu of *Lactobacillus plantarum* IFPL935. Finally, a 1-week wash-out period was included at the end of the experiment for both systems. During the whole study, samples were collected at regular time points from the three colon vessels and stored at

−20 °C until further analysis, excepting for microbiological plate counts that were performed at the time of sampling.

V.3.2 Bacterial culture conditions and plate counts

Lactobacillus plantarum IFPL935 (CECT 4599) was routinely grown aerobically at 37 °C in MRS broth. To reach the daily supplementation of 1010 cfu of IFPL935, 10 ml of *L. plantarum* IFPL953 grown to 109 cfu/ml were added daily to the stomach compartment in one of the SHIME systems, right after the addition of the red wine extract. Numbers of lactobacilli in the inoculum were counted using MRS-agar plates and in the AC, TC and DC vessels using LAMVAB agar (Hartemink et al. 1997), and plates were incubated aerobically at 37 °C for 48 h. Total aerobes and total anaerobes were determined by plating on BHI agar and incubation at 37 °C for 24 h aerobically or 72 h anaerobically, respectively. Total coliforms were enumerated in MacConkey agar incubated for 24 h aerobically at 37 °C.

V.3.3 DNA extraction and analysis

Bacterial DNA was extracted using hexadecyltrimethylammonium bromide (CTAB) buffer and phenol-chloroform-isoamyl alcohol and bead-beating, as described previously (Griffiths et al. 2000; Kowalchuk et al. 1998). The DNA was precipitated with polyethelene glycol (PEG-6000), washed in ice cold 70% ethanol and dried in a Speed-Vac, prior to resuspension in distilled water. The concentration and quality of the samples were assessed with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

V.3.4 Quantitative PCR (qPCR)

Bacterial numbers in the AC, TC and DC vessels were quantified by qPCR using SYBR green methodology (Kappa Biosystems, Woburn,

MA, USA) with the IQ5 Multicolor Real-Time PCR Detection System and data analyses (Bio-Rad Laboratories Inc., Hercules, CA, USA). The bacterial groups targeted for qPCR were total bacteria, *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterobacteriaceae*, and the specific phylogenetic groups *Blautia coccoides-Eubacterium rectale* Cluster XIVa, *Ruminococcus* Cluster IV, and *Clostridium leptum* subgroup specific cluster IV. Butyrate-producing bacteria were also estimated by quantifying the gene encoding butyryl-CoA:acetate CoA transferase (BcoAT). Primers, amplification conditions and calculation of copy numbers have been detailed previously (Barroso et al. 2013). DNA from *Escherichia coli* DH5α, *L. plantarum* IFPL935, *Bifidobacterium breve* 29M2 and *Bacteroides fragilis* DSM2151 was used for quantification of total bacteria, *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, respectively. For the rest of groups analyzed, samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA), as described previously (Barroso et al., 2013).

V.3.5 PCR-DGGE

The diversity of the *Lactobacillus* community in the AC, TC and DC vessels and the presence of *L. plantarum* were assessed by PCR-DGGE and using the primers Lab-159F and Uni-515-GC-R, as described by Heilig et al. (2002). DGGE was performed with a DCode system (Bio-Rad) using a 9% polyacrylamide gel with a 30–50% gradient of 7 M urea and 40% formamide. The obtained band patterns were analyzed using InfoQuest FP software version 5.1 (Bio-Rad). Clustering was performed with Pearson correlation and the UPGMA method.

V.3.6 Bacterial metabolism

V.3.6.1 Analysis of short and branched-chain fatty acids (SCFA and BCFA), lactic acid and ammonium

The SCFA and BCFA were extracted from the samples with diethyl ether, after the addition of 2-methyl hexanoic acid as an internal standard and extracts were analysed by GC as described previously (Possemiers et al. 2004). SCFA were separated using a capillary free fatty acid packed column (EC-1000 Econo-Cap, 25 m × 0.53 mm x 1.2 µm) and detected with a flame ionization detector. Nitrogen was used as carrier gas. Lactic acid was measured spectrometrically with an enzymatic D-/L-lactic acid Kit (R-Biopharm, Darmstadt, Germany), according to the manufacturer's instructions.

Ammonium was released from samples as ammonia by addition of MgO and distillation into boric acid-indicator solution using an autodistillation Vapodest 30´ (Gerhardt Analytical Systems, Brackley Northants, UK), as earlier described by Bremner and Keeney (1965). Ammonia was determined by titration with standard HCl using a 685 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium).

V.3.6.2 Analysis of phenolic metabolites

Phenolic metabolites were analyzed by a previous UPLC-ESI-MS/MS method (Sánchez-Patán et al. 2011) further implemented (Jiménez-Girón et al. 2013). The liquid chromatographic system was a Waters Acquity UPLC (Milford, MA, USA) equipped with a binary pump, an autosampler thermostated at 10 ºC, and a heated column compartment (40 ºC). The column employed was a BEH-C18, 2.1 x 100 mm and 1.7 µm particle size from Waters (Milford, MA, USA). The mobile phases were 2% acetic acid in water (A) and 2% acetic acid in acetonitrile (B). The gradient program was as follows: 0 min, 0.1% B; 1.5 min, 0.1%

B; 11.17 min, 16.3% B; 11.5 min, 18.4% B; 14 min, 18.4% B; 14.1 min, 99.9% B; 15.5 min, 99.9% B; 15.6 min, 0.1% B. Equilibrium time was 2.4 min resulting in a total runtime of 18 min. The flow rate was set constant at 0.5 ml/min and injection volume was 2 µl. The LC effluent was pumped to an Acquity TQD tandem quadrupole mass spectrometer equipped with a Z-spray electrospray ionization (ESI) source operated in negative polarity mode. The ESI parameters were set as follows: capillary voltage, 3 kV; source temperature, 130 ºC; desolvation temperature, 400 ºC; desolvation gas (N2) flow rate, 750 l/h; cone gas (N2) flow rate, 60 l/h. The ESI was operated in negative ionization mode. For quantification purposes, data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific to each compound. The MS/MS parameters (cone voltage, collision energy and MRM transition) of the 60 phenolic compounds targeted in the present study (mandelic acids, benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, cinnamic acids, 4-hydroxyvaleric acids and valerolactones) were previously reported (Jiménez-Girón et al. 2013). All metabolites were quantified using the calibration curves of their corresponding standards, except for 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric, and 4-hydroxy-5-(phenyl)-valeric acids) which were quantified using the calibration curves of 3-(3',4'-dihydroxyphenyl)-propionic, and propionic acids, respectively. Data acquisition and processing was realized with MassLynx 4.1 software.

V.3.7 Statistical analysis

Mean values, standard deviations and correlation coefficients were calculated on the basis of the values for the different variables during the incubation period (microbial groups, SCFAs, acetate, propionate, butyrate, ammonium and phenolic metabolites). Analysis of variance (ANOVA) was used for multiple comparisons of the different variables taken into account different factors (time, compartments, and addition of

L. plantarum IFPL935) ($P < 0.05$). All statistical analyses were carried out using the STATISTICA program for Windows, version 7.1 (StatSoft. Inc. 1984−2006, www.statsoft.com).

V.4 RESULTS

V.4.1 Microbiological changes

The colonic microbiota was allowed to reach the steady-state conditions during the 3-weeks period of stabilization in each of the three colon vessels of the Twin-SHIME systems (Day 0, Table V.1). After this stabilization period, impact of daily feeding with either the phenolic extract or the phenolic extract and *L. plantarum* IFPL935 on log numbers of total bacteria, *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Enterobacteriaceae*, is shown in Table V.1. Figure V.1 shows the log numbers for the butyrate- producing phylogenetic groups *B. coccoides-E. rectale* Cluster XIVa, *C. leptum* subgroup specific Cluster IV, and *Ruminococcus* Cluster IV. Feeding the SHIME with the phenolic extract caused a decrease of approx. 1 log numbers of total bacteria in the AC vessels after 24 h treatment (Table V.1). This initial decrease of numbers affected mostly to the counts of *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Enterobacteriaceae* (Table V.1) and *B. coccoides/E. rectale* (Fig. V.1). In most cases, bacterial numbers were readily recovered after the first week of treatment. The largest reduction in numbers was recorded for *Bacteroides*, showing a decrease of 2 log numbers in the AC vessels at the first day of treatment. The inhibitory effect of the red wine extract was less pronounced but more persistent for *Bifidobacterium*, which numbers continuously fell down during the first week of treatment and did not recover until the second week. Addition of *L. plantarum* IFPL935 to one of the Twin-SHIME systems together with the phenolic extract resulted in relative stable numbers throughout the treatment of

total bacteria, *Lactobacillus* and *Enterobacteriaceae* (Table V.1). In these bacterial groups, no impact of phenolic extract on the log numbers after 1 day of treatment was observed with the presence of *L. plantarum* IFPL935. The diversity of lactobacilli in the AC, TC and DC vessels was followed by PCR-DGGE targeting the *Lactobacillus* genus. The cluster analysis of the DGGE profiles grouped the samples during the treatment period mainly based on the addition of *L. plantarum* IFPL935 to the SHIME feeding medium (Fig. S1 in the supplemental material). Additionally, agar plate counts revealed higher lactobacilli values during the treatment and first days of wash out in the colonic vessels of the SHIME unit supplemented with *L. plantarum* IFPL935 (results not shown).

Table V.1. Mean (SD) q-PCR counts (log copy number/ml) of bacterial groups in the ascending (AC), transverse (TC) and descending colon (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (Provin) and the extract supplemented with *L. plantarum* IFPL935 (Provin+Lp935) and the was out period

Bacteria	Colonic vessel	Treatment	Intervention period				
			Day 0 (steady state)	Day 1	Week 1	Week 2	Week 3 (wash out)
Total counts	AC	Provin	8.28 (0.32)	7.20 (0.08)	8.28 (0.34)	7.86 (0.29)	8.03 (0.21)
		Provin+Lp935	8.17 (0.06)	8.66 (0.07)	8.26 (0.38)	8.00 (0.31)	7.99 (0.14)
	TC	Provin	8.60 (0.12)	8.49 (0.03)	8.20 (0.38)	8.06 (0.50)	8.22 (0.30)
		Provin+Lp935	8.42 (0.30)	8.00 (0.10)	8.23 (0.43)	8.19 (0.11)	8.06 (0.15)
	DC	Provin	8.43 (0.13)	8.33 (0.07)	8.42 (0.30)	8.34 (0.50)	8.37 (0.44)
		Provin+Lp935	8.34 (0.25)	8.08 (0.05)	8.57 (0.14)	8.33 (0.06)	8.14 (0.42)
Lactobacillus	AC	Provin	7.79 (0.41)	6.30 (0.16)	7.09 (0.29)	7.12 (0.67)	7.29 (0.30)
		Provin+Lp935	7.57 (0.62)	7.62 (0.34)	7.40 (0.13)	7.16 (0.57)	7.69 (0.15)
	TC	Provin	7.63 (0.11)	7.16 (0.16)	6.76 (0.20)	7.29 (0.25)	7.31 (0.18)
		Provin+Lp935	7.51 (0.13)	6.94 (0.37)	7.44 (0.05)	7.74 (0.24)	7.54 (0.29)
	DC	Provin	7.32 (0.06)	6.89 (0.02)	6.87 (0.10)	7.21 (0.41)	7.33 (0.35)
		Provin+Lp935	7.44 (0.12)	7.03 (0.31)	7.11 (0.12)	7.55 (0.16)	7.42 (0.41)
Bifidobacterium	AC	Provin	6.74 (0.40)	6.04 (0.20)	5.91 (0.68)	6.94 (0.21)	6.50 (0.80)
		Provin+Lp935	6.73 (0.60)	6.36 (0.54)	5.44 (0.91)	6.74 (0.67)	5.96 (0.63)
	TC	Provin	6.97 (0.17)	7.01 (0.30)	6.57 (0.22)	7.31 (0.58)	7.17 (0.87)
		Provin+Lp935	6.72 (0.15)	6.75 (0.23)	6.07 (0.17)	7.23 (0.26)	6.86 (0.70)
	DC	Provin	7.03 (0.45)	6.69 (0.39)	6.43 (0.17)	7.23 (0.43)	6.92 (0.82)
		Provin+Lp935	6.69 (0.29)	6.52 (0.65)	6.47 (0.10)	7.13 (0.46)	6.92 (0.73)
Bacteroides	AC	Provin	8.23 (0.07)	6.20 (0.05)	8.09 (0.52)	8.28 (0.08)	8.33 (0.32)
		Provin+Lp935	8.20 (0.46)	6.23 (0.01)	8.27 (0.32)	8.11 (0.01)	8.46 (0.26)
	TC	Provin	8.47 (0.12)	7.95 (0.02)	8.28 (0.52)	8.27 (0.35)	8.37 (0.24)
		Provin+Lp935	8.32 (0.23)	7.19 (0.07)	8.35 (0.53)	8.32 (0.08)	8.39 (0.49)
	DC	Provin	8.31 (0.14)	7.98 (0.03)	8.49 (0.24)	8.04 (0.40)	8.41 (0.25)
		Provin+Lp935	8.37 (0.13)	7.49 (0.01)	8.68 (0.34)	8.04 (0.47)	8.00 (0.28)
Enterobacteriaceae	AC	Provin	7.35 (0.21)	6.37 (0.21)	7.34 (0.33)	6.59 (0.73)	7.28 (0.19)
		Provin+Lp935	7.15 (0.20)	7.06 (0.23)	7.52 (0.55)	6.44 (0.35)	7.25 (0.10)
	TC	Provin	7.63 (0.09)	7.40 (0.02)	7.31 (0.23)	7.15 (0.51)	7.35 (0.12)
		Provin+Lp935	7.54 (0.26)	6.84 (0.24)	7.09 (0.29)	7.16 (0.29)	7.26 (0.27)
	DC	Provin	7.44 (0.17)	7.43 (0.23)	7.04 (0.54)	7.31 (0.34)	7.35 (0.09)
		Provin+Lp935	7.40 (0.29)	7.01 (0.22)	6.69 (0.76)	7.25(0.28)	7.13 (0.42)

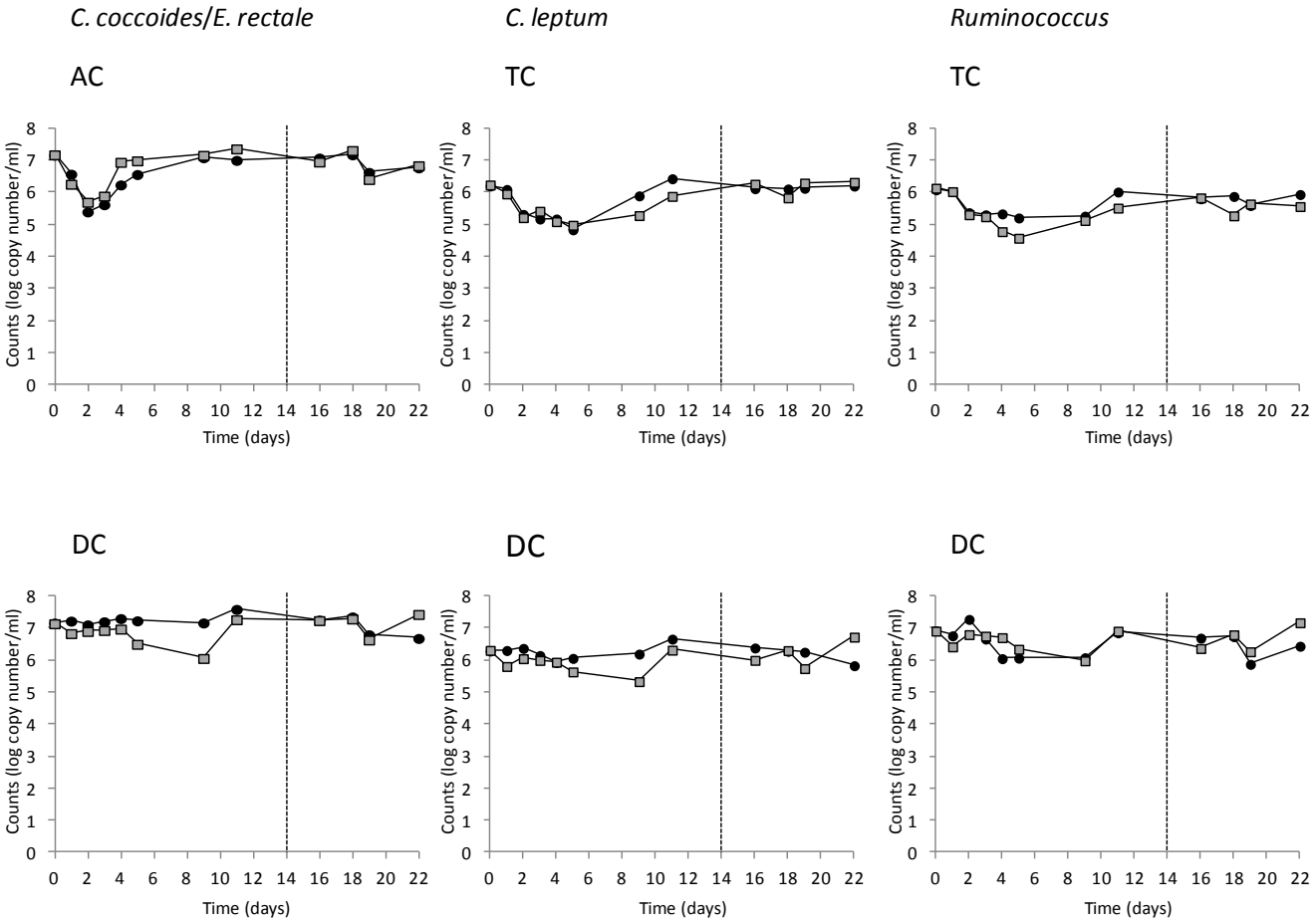

Figure V.1. Changes in q-PCR counts (log copy number/ml) of butyrate-producing groups belonging to *Clostridium* clusters IV and XIVa in the ascending (AC), transverse (TC) and descending colon (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey squares) and the extract supplemented with *L. plantarum* IFPL935 (black circles) and the was out period. The dotted line indicates the end of the polyphenol treatment

The numbers of the butyrate-producing bacteria group *B. coccoides-E. rectale* was equally represented in the three colonic vessels at the start of the treatment (about 7 log), whereas *C. leptum* and *Ruminococcus* prevailed in TC and DC vessels (about 6-7 log) (Fig. V.1), being almost under the detection limit in the AC vessels. Impact of feeding the Twin-SHIME units with the phenolic extract on these three bacterial groups was more pronounced in the AC vessels for *B. coccoides-E. rectale* and in the TC vessels for *C. leptum* and *Ruminococcus*, where the effects appeared earlier in time. The BCoAT gene numbers corresponded with the results of butyrate producing bacteria counts, showing higher values in the TC and DC vessels than in the AC ones and, particularly, in the DC from the SHIME unit supplemented with *L. plantarum* IFPL935 (results not shown).

V.4.2 Wine polyphenols supplementation affects microbial metabolism

Evolution of the microbial metabolism (fermentation and proteolysis) expressed as the content in SCFA, lactic acid, BCFA and ammonium is shown in Fig. V.2. Daily intake of the polyphenolic extract caused a sharp decrease in both fermentation and proteolysis measured during the first days of treatment in all the AC, TC and DC vessels. Proteolysis rate measured by ammonium and BCFA content showed the minimum values in all vessels during the first week of treatment, particularly for ammonium. No differences were observed between both treatments with and without *L. plantarum* IFPL935 (Fig. V.2A). Regarding the fermentation profile, acetic and propionic acid values decreased similarly, reaching minimum values in all three vessels during the first three days for both treatments (Fig. V.2B). Butyric acid was lessened to no detectable levels during the first two days of treatment in all the vessels excepting the DC and TC vessels added with phenolic extract and *L. plantarum* IFPL935 (Fig. V.2C). On the other hand, the lactic acid

content was not negatively affected by any of the treatments. Moreover, the AC vessel of the SHIME supplemented with the phenolic extract and *L. plantarum* IFPL935 showed an increase of lactic acid at the starting of the intervention period and a decrease associated to the recovery of butyrate content to initial levels (Fig. V.2C). The addition of *L. plantarum* IFPL935 also caused differences in the production of butyric acid in the DC vessels where the highest values for this acid were found. In general, the microbial metabolic activity present at the start of the feeding was recovered in all vessels for both treatments after 5 days of the experiments, except for the production of butyric acid in the DC vessel without *L. plantarum* IFPL935, which only returned to initial values at the end of the wash-out period (Fig. V.2C).

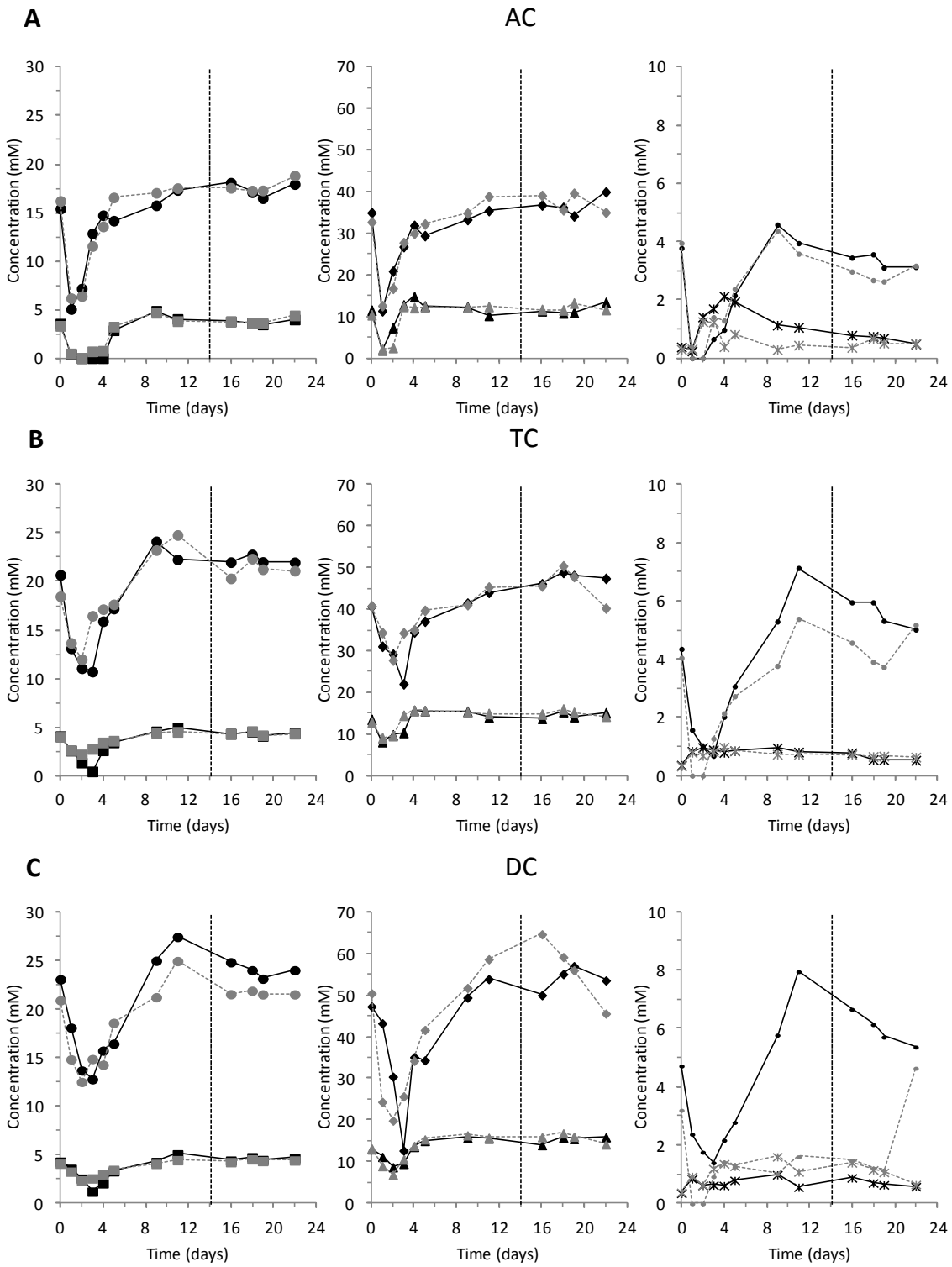

Figure V.2. Changes in concentration (mM) of ammonium (circles), BCFA (squares), acetic acid (diamonds), propionic acid (triangles), butyric acid (dots) and lactic acid (asterisks) in the ascending (AC; A), transverse (TC; B) and descending colon (DC; C) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey symbols) and the extract supplemented with *L. plantarum* IFPL935 (black symbols) and the was out period. The vertical dotted line indicates the end of the polyphenol treatment

V.4.3 Changes in phenolic metabolism

Within the 60 phenolic metabolites targeted, a total of 26 compounds including benzoic acids, phenols, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones and cinnamic acids, exhibiting different substitutions in the aromatic ring, were quantified in the AC, DC and TC contents during the 2-weeks period of treatments and the wash-out period (Table S1 in the supplemental material). Formation of these metabolites was in accordance with disappearance of wine phenolic precursors (Table S2 in the supplemental material), mainly flavan-3ols (monomers and dimeric procyanidins), but also anthocyanins, flavonols and stilbenes. To better summarize these changes, Fig. V.3 displays the sum of concentrations of the precursor monomers (+)-catechin and (-)-epicatechin, and procyanidins B1, B2, B3, B4 (Fig. V.3A), the intermediate metabolites phenyl-γ-valerolactones and phenylvaleric acid derivatives (Fig. V.3B) and the phenylpropionic and phenylacetic acid derivatives (Fig. V.3C) during the continuous feeding of the Twin-SHIME with the red wine polyphenolic extract.

Flavan-3-ol monomers and procyanidins were mainly detected in AC vessels and starting from day 2 of the wine polyphenol treatment. The concentration of these precursor compounds in the AC vessels increased during the intervention, followed by a return to baseline during the wash-out period (Figure V.3A). Precursors were also detected in the TC compartments, but their concentration diminished earlier than in the AC vessels. Interestingly, the content of these flavan-3-ol monomers and procyanidins in TC and DC vessels were lower in the SHIME unit supplemented with *L. plantarum* IFPL935.

The formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid, intermediate metabolites arising from first steps in the microbial degradation of flavan-3-ols, was mainly observed in the TC vessels and their concentration increased after

day 4 of both treatments (Fig. V.3B). These compounds were also present in the DC vessels but to a much lesser extent and only in those with no addition of *L. plantarum* IFPL935. In accordance with these results, 4-hydroxy-5-(phenyl)-valeric acid content increased with time and was mainly detected in the DC vessels (Table S1 in the supplemental material).

Also, and as derived from galloylated flavan-3-ols, an increase in the content of gallic acid was only detected in the AC vessels, whereas 3-O-methylgallic acid was also measured in the TC vessels (Table S1 and Fig S2 in the supplemental material). Likewise, pyrogallol formation was only observed in the AC vessels whereas pyrocatechol was measured mainly in the AC and TC compartments. Supplementation with *L. plantarum* IFPL935 did not cause differences in the metabolism of these compounds.

The evolution of 3-(4´-hydroxyphenyl) propionic acid, 4-hydroxyphenylacetic acid and phenylacetic acid, which can also be derived from aromatic amino acid catabolism, followed the same trends as the microbial metabolites released from proteolysis depicted in Fig. V.2A (Table S1 in the supplemental material). The extent of formation of microbial phenolic metabolites from the flavan-3-ol compounds was estimated in the SHIME vessels during the treatment and wash-out periods by computing the formation of phenylpropionic acids [3-(3´,4´-dihydroxyphenyl)-propionic, 3-(3´-hydroxyphenyl)-propionic, and 3-phenylpropionic] and phenylacetic acids [3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic] derivatives (Fig. V.3C). The highest values of these metabolites were observed in the DC vessels, being still detected during the wash-out period. In the SHIME unit supplemented with *L. plantarum* IFPL935 the phenylpropionic acid derivatives, mainly represented by 3-(3´-hydroxyphenyl)propionic acid, appeared at earlier

stages than the phenylacetic acid metabolites and their concentration was the highest when comparing values from the two SHIME units.

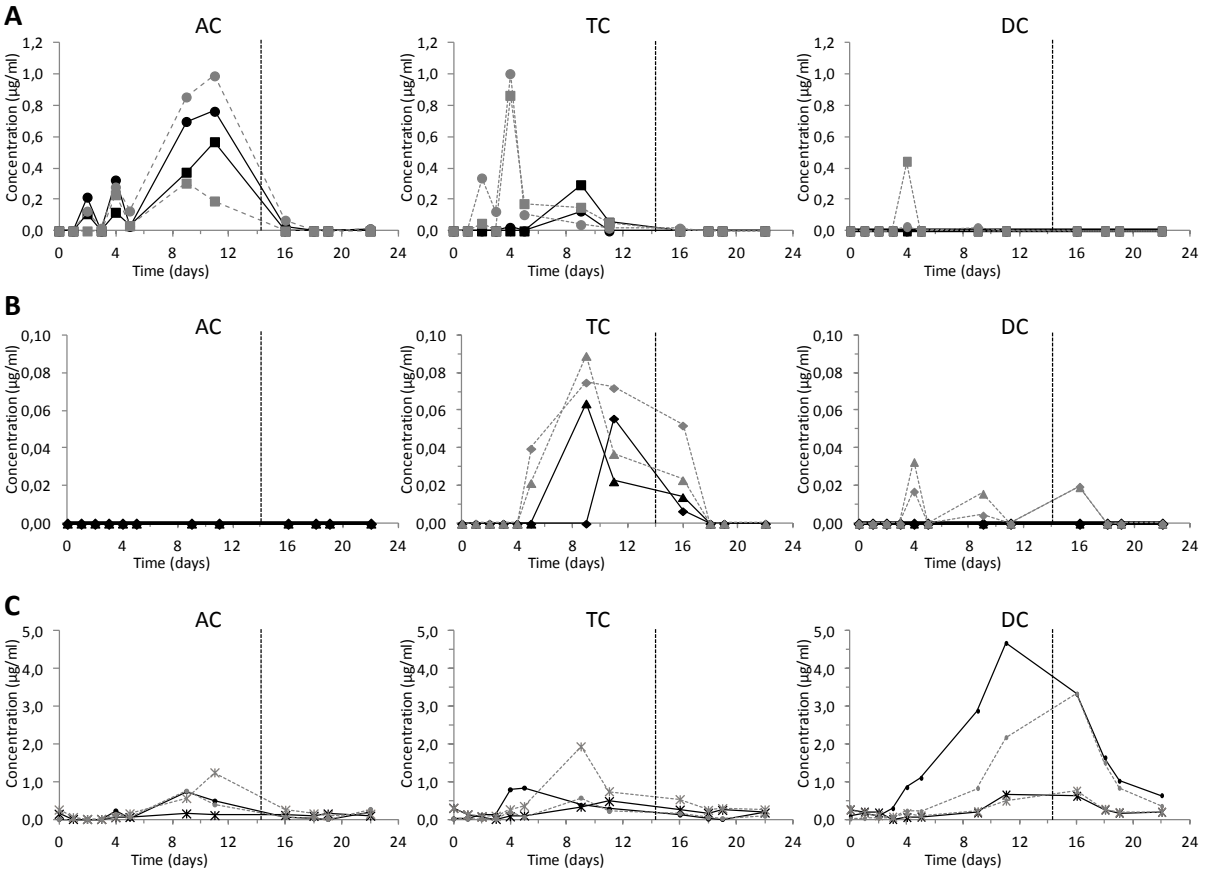

Figure V.3. Changes in concentration (µg/ml) of the sum of (A) precursor monomers (+)-catechin and (-)-epicatechin (circles) and procyanidins B1, B2, B3, B4 (squares), (B) the intermediate metabolites phenyl-γ-valerolactones (diamonds) and phenylvaleric acid derivatives (triangles) and (C) the phenylpropionic (dots) and phenylacetic acid (asterisks) derivatives in the ascending (AC), transverse (TC) and descending colon (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey symbols) and the extract supplemented with *L. plantarum* IFPL935 (black symbols) and the was out period. The vertical dotted line indicates the end of the polyphenol treatment.

V.5 DISCUSSION

The human gut microbiota contributes to many of the important host metabolic functions, including the increase of bioavailabilityof dietary polyphenols (van Duynhoven et al. 2011). Conversely, polyphenols and their resultant metabolites can modify the gut bacterial population composition and activity (Requena et al. 2010). Most of the studies that have examined the antimicrobial effect of dietary polyphenols have focused on single polyphenol molecules and/or single strains. In this work, we have studied the effect on the gut microbiota of the long-term feeding with a red wine polyphenolic extract and the impact of the supplementation of *L. plantarum* IFPL935, previously characterized as a flavan-3-ol metabolizer (Barroso et al. 2013; Sánchez-Patán et al. 2012b). We have conducted the study in the *in vitro* model of the human intestinal microbiota SHIME, which has demonstrated to allow nutritional interventions with repeated doses to evaluate long-term effect of food ingredients on the microbiota allocated in three consecutive colon regions (Grootaert et al. 2009; Marzorati et al. 2010; Van de Wiele et al. 2007; Van den Abbeele et al. 2013).

The present study shows that the initial addition of red wine polyphenols exerted an overall antimicrobial effect on the gut microbiota in the AC, but the effect disappeared during continuous feeding with polyphenols, probably due to the onset of microbial metabolism of polyphenols. The antimicrobial effect of red wine and grape polyphenolic extracts following either a single dose or a continuous feeding of the SHIME has recently been described (Kemperman et al. 2013). These authors reported a more pronounced antimicrobial effect on the colonic microbiota, which did not fully recover until the wash-out period. Differences between studies could be due to the fact that the amount of polyphenolic compounds daily supplemented by Kemperman et al. (2013)

was 5-times higher than in the present study. Besides, grape extracts have demonstrated higher antimicrobial capacity than extracts obtained from red wine (Cueva et al. 2013), probably because of a higher content of proanthocyanidins and gallate derivatives (Xia et al. 2010).

Among polyphenols, flavan-3-ols and flavonols have received most attention due to their wide spectrum and higher antimicrobial activity in comparison with other polyphenols (Daglia 2012). In our study, all bacterial groups analyzed in the AC vessels were initially affected by the supplementation with red wine polyphenols. Among them, *Bacteroides* and the butyrate-producers *B. coccoides/E. rectale* were the most affected groups, whereas *Bifidobacterium* showed a more persistent effect. *Bacteroides* and *Bifidobacterium* have been described previously (Kemperman et al. 2013) as the genera most affected after feeding the SHIME colonic microbiota with black tea and red wine polyphenols. Additionally, intake of flavan-3-ol-rich sources for prolonged periods of time has generally correlated with the decrease of *Clostridium* groups (Dolara et al. 2005; Tzounis et al. 2011; Wu et al. 2011). On the other hand, counts of lactobacilli are generally unaffected or moderately increase after intake of diets enriched in flavanols (Tzounis et al. 2011). Nevertheless, a different sensitivity to the presence of flavan-3-ols in the growth medium has been described for several *Lactobacillus* species (Tabasco et al. 2011), ranging from the optimal growth observed for *L. plantarum*, L. casei, and L. bulgaricus strains and the growth inhibition of *L. fermentum*, *L. acidophilus* and *L. vaginalis*. Among the strains assayed, however, only *L. plantarum* IFPL935 was able to metabolize flavan-3-ol compounds (Fernández-Patán, 2012b; Tabasco et al. 2011).

Although the antimicrobial effect caused by daily feeding the SHIME with red wine polyphenols was predominantly observed during the first 1-2 days of treatment and it was more distinguished in the AC vessels, the inhibitory effect on the overall microbial metabolism,

however, was similar in the three vessels and it lasted for the first 5 days of treatment (Fig. V.2). These results indicate a more pronounced effect of the polyphenol compounds in the microbial functionality than in its viability. The initial exposure to polyphenols may up-regulate polyphenol-induced stress responses related to defensive mechanisms while simultaneously down-regulating various metabolic functions, for example, carbon and energy metabolism (Stevenson and Hurst 2007). Decreased rate of microbial fermentation and proteolysis, determined as reduction in the formation of SCFA and BCFA, is associated with high tannin-rich feeds intake in ruminant livestock (Bodas et al. 2012). The effects of some plant extracts have been described to last only a few days, indicating that ruminal microorganisms adapt to the compounds with time (Busquet et al. 2005). Similarly, the regular feeding of the SHIME with a red wine polyphenolic extract was able to introduce microbial and metabolic changes until the microbiota adapted its metabolism and returned to steady state similar conditions during the treatment.

In terms of microbial metabolism, the addition of *L. plantarum* IFPL935 caused an increase in the formation of lactic acid at the start of the polyphenol treatment in the AC vessel and of butyric acid in the TC and DC vessels after the day 5 of treatment (Fig. V.2). The increased formation of butyric acid in the DC vessel supplemented with *L. plantarum* IFPL935 was also associated with a lower content of lactic acid. Both phenomena can partially be explained by the recovery at this point of the counts of butyrate-producing bacteria from clostridial cluster XIVa and eventually by bacterial cross-feeding production of butyric acid from lactic acid. In fact, the only vessels where the content of butyric acid did not decrease to zero values at the start of the polyphenol treatment were the TC and DC vessels of the SHIME supplemented with *L. plantarum* IFPL 935 (Fig. V.2). Duncan et al. (2004) reported that most of the faecal species that utilize lactate to produce butyrate via the butyryl CoA:acetate CoA transferase route, belong to the clostridial cluster XIVa

(*Lachnospiraceae*). The results obtained in this study confirm the previously observed effect of *L. plantarum* IFPL935 to increase the formation of butyric acid during batch incubations with different colonic microbiota (Barroso et al. 2013). This compound has been described as beneficial for human health since low prevalence in the gut has been associated with chronic immune or metabolic-related diseases including inflammatory bowel disease (Frank et al. 2007) and obesity (Le Chatelier et al. 2013).The capability of *L. plantarum* IFPL935 to initiate the catabolism of polyphenols either via galloyl-esterase, decarboxylase and benzyl alcohol dehydrogenase activities (Tabasco et al. 2011) or mostly by the cleavage of the heterocyclic ring of monomeric flavan-3-ols (Sanchez-Patán et al. 2012b), might have contributed to diminish the inhibitory effect of the red wine extract when both were added to the SHIME units. So far and besides this strain, only a few bacterial strains, such as *Eubacterium* sp. SDG-2, *Eggerthella* sp. CAT-1, *Eggerthella lenta* rK3, and *Flavonifractor plautii* aK2 have been reported to be able to initiate the metabolism of flavanol-3-ols by cleavage of the heterocyclic C-ring (Jin and Hattori 2012; Kutschera et al. 2011; Wang et al. 2001).

A lower concentration of monomeric flavan-3-ols and first and intermediate metabolites (5-(3′-hydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid), mainly represented in the AC and TC vessels, was observed in the vessels of the SHIME unit supplemented with *L. plantarum* IFPL935. In addition, further metabolism of the polyphenol intermediate compounds to the formation of phenylpropionic acids was detected earlier and reached higher concentrations in the DC vessel containing the strain when compared to the other colonic vessels (Fig. V.3C). For both treatments, bacterial conversion of red wine polyphenols was more favorable in the distal colon, which is in agreement with previous studies where a higher microbial conversion of phenolic compounds was found in the distal compartments (Barroso et al. 2013; Van Dorsten et al. 2012).

Phenyl propionic acid and 3-(3´-hydroxyphenyl)propionic acid were the microbial metabolites that accounted most for the differences between treatments. Phenylpropionic acid derivatives are considered to arise from β-oxidation of phenylvaleric acid derivatives (Monagas et al. 2010), except for 3-(4´-hydroxyphenyl)propionic acid that can result from the microbial catabolism of tyrosine (Russell et al. 2013). The evolution of this compound during the SHIME treatment with red wine polyphenols indicated that 3-(4´-hydroxyphenyl)propionic acid aroused mostly from amino acid catabolism (Table S1 in the supplemental material). The phenyl propionic metabolite produced at a higher concentration was 3-(3´-hydroxyphenyl) propionic acid, which has been identified as a strong urinary marker of red wine and grape extracts intake (Jacobs et al. 2012; Ward et al. 2004). This microbial metabolite has been shown to reduce the inflammatory response of human peripheral blood mononuclear cells stimulated with lipopolysaccharide (Monagas et al. 2009) and to have anti-thrombotic activity (Rechner and Kroner 2005). In conclusion, this study provides data supporting the potential use of *L. plantarum* IFPL935 to increase gut butyrate formation and favour polyphenol metabolism in an *in vitro* gastro intestinal system. This opens possibilities of using the strain as a food ingredient for helping individuals showing a low polyphenol-fermenting metabotype (Bolca et al. 2013) to increase their gut capacities of metabolizing dietary polyphenols.

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V.6 REFERENCES

Arranz S, Chiva-Blanch G, Valderas-Martínez P, Medina-Remón A, Lamuela-Raventós RM, Estruch R (2012) Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer. Nutrients 4:759–781

Barroso E, Sánchez-Patán F, Martín-Alvarez PJ, Bartolomé B, Moreno-Arribas MV, Peláez C, Requena T, Van de Wiele T, Martínez-Cuesta MC (2013) *Lactobacillus plantarum* IFPL935 favors the initial metabolism of red wine polyphenols when added to a colonic microbiota. J Agric Food Chem 61:10163–10172

Bodas R, Prieto N, García-González R, Andrés S, Giráldez FJ, Lopez S (2012) Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim Feed Sci Technol 176: 78–93

Bolca S, Van de Wiele T, Possemiers S (2013) Gut metabotypes govern health effects of dietary polyphenols. Curr Opin Biotechnol 24:220–225

Bremner JM, Keeney RD (1965) Steam distillation methods for determination of ammonium, nitrate and nitrite. Anal Chem Acta 32:485−495

Busquet M, Calsamiglia S, Ferret A, Kamel C (2005) Screening for effects of plant extracts and active compounds of plants on dairy cattle rumen microbial fermentation in a continuous culture system. Anim Feed Sci Technol 124:597–613

Cueva C, Sánchez-Patán F, Monagas M, Walton GE, Gibson GR, Martín-Álvarez PJ, Bartolomé B, Moreno-Arribas MV (2013) *In vitro* fermentation of grape seed flavan-3-ol fractions by human faecal microbiota: changes in microbial groups and phenolic metabolites. FEMS Microbiol Ecol 83:792–805

Daglia M (2012) Polyphenols as antimicrobial agents. Curr Opin Biotechnol 23:174–181

De Boever P, Deplancke B, Verstraete W (2000) Fermentation by gut microbiota cultured in a Simulator of the Human Intestinal Microbial Ecosystem is improved by supplementing a soygerm powder. J Nutr 130:2599–2606

Dolara P, Luceri C, De FC, Femia AP, Giovannelli L, Caderni G, Cecchini C, Silvi S, Orpianesi C, Cresci A (2005) Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of colonic mucosa in F344 rats. Mutat Res 591:237−246

Duncan SH, Louis P, Flint HJ (2004) Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 70:5810–5817

Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci USA 104:13780–13785

Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA based microbial community composition. Appl Environ Microbiol 66:5488−5491

Grootaert C, Van den Abbeele P, Marzorati M, Broekaert WF, Courtin CM, Delcour JA, Verstraete W, Van de Wiele T (2009) Comparison of prebiotic effects of arabinoxylan oligosaccharides and inulin in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 69:231–242

Hartemink R, Domenech VR, Rombouts FM (1997) LAMVAB - A new selective medium for the isolation of lactobacilli from faeces. J Microbiol Meth 29:77–84

Heilig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD, de Vos WM (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol 68:114–123

Jacobs DM, Fuhrmann JC, van Dorsten FA, Rein D, Peters S, van Velzen EJ, Hollebrands B, Draijer R, van Duynhoven J, Garczarek U (2012) Impact of short-term intake of red wine and grape polyphenol extract on the human metabolome. J Agric Food Chem 60:3078–3085

Jiménez-Girón A, Queipo-Ortuño MI, Boto-Ordóñez M, Muñoz-González I, Sánchez-Patán F, Monagas M, Martín-Álvarez PJ, Murri M, Tinahones FJ, Andrés-Lacueva C, Bartolomé B, Moreno-Arribas MV (2013) Comparative study of microbial-derived phenolic metabolites in human feces after intake of gin, red wine, and dealcoholized red wine. J Agric Food Chem 61:3909−3915

Jin JS, Hattori M (2012) Isolation and characterization of a human intestinal bacterium *Eggerthella* sp. CAT-1 capable of cleaving the C-ring of (+)-catechin and (-)-epicatechin, followed by p-dehydroxylation of the B-ring. Biol Pharm Bull 35:2252–2256

Kemperman RA, Gross G, Mondot S, Possemiers S, Marzorati M, Van de Wiele T, Dore J, Vaughan EE (2013) Impact of polyphenols from black tea and red wine/grape juice on a gut model microbiome. Food Res Int 53:659–669

Kishimoto Y, Tani M, Kondo K (2013) Pleiotropic preventive effects of dietary polyphenols in cardiovascular diseases. Eur J Clin Nutr 67:532–535

Kowalchuk GA, Bodelier PLE, Heilig GHJ, Stephen JR, Laanbroek HJ (1998) Community analysis of ammonia oxidizing bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol Ecol 27:339−350

Kutschera M, Engst W, Blaut M, Braune A (2011) Isolation of catechin-converting human intestinal bacteria. J Appl Microbiol 111:165−175

Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, Almeida M, Arumugam M, Batto JM, Kennedy S, Leonard P, Li J, Burgdorf K, Grarup N, Jørgensen T, Brandslund I, Nielsen HB, Juncker AS, Bertalan M, Levenez F, Pons N, Rasmussen S, Sunagawa S, Tap J, Tims S, Zoetendal EG, Brunak S, Clément K, Doré J, Kleerebezem M, Kristiansen K, Renault P, Sicheritz-Ponten T, de Vos WM, Zucker JD, Raes J, Hansen T, MetaHIT consortium, Bork P, Wang J, Ehrlich SD, Pedersen O (2013) Richness of human gut microbiome correlates with metabolic markers. Nature 500:541–546.

Marzorati M, Verhelst A, Luta G, Sinnott R, Verstraete W, Van de Wiele T, Possemiers S (2010) *In vitro* modulation of the human gastrointestinal microbial community by plant-derived polysaccharide-rich dietary supplements. Int J Food Microbiol 139:168–176

Monagas M, Khan N, Andrés-Lacueva C, Urpí-Sardá M, Vázquez-Agell M, Lamuela-Raventós RM, Estruch R (2009) Dihydroxylated phenolic acids derived from microbial metabolism reduce lipopolysaccharide-stimulated cytokine secretion by human peripheral blood mononuclear cells. Br J Nutr 102:201–206

Monagas M, Urpi-Sarda M, Sánchez-Patán F, Llorach R, Garrido I, Gómez-Cordovés C, Andrés-Lacueva C, Bartolome B (2010) Insights into

the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. Food Funct 1:233−253

Possemiers S, Verthé K, Uyttendaele S, Verstraete W (2004) PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol 49:495–507

Queipo-Ortuño MI, Boto-Ordóñez M, Murri M, Gómez-Zumaquero JM, Clemente-Postigo M, Estruch R, Cardona Díaz F, Andrés-Lacueva C, Tinahones FJ (2012) Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. Am J Clin Nutr 95:1323−1334

Rechner AR, Kroner C (2005) Anthocyanins and colonic metabolites of dietary polyphenols inhibit platelet function. Thromb Res 116:327–334

Requena T, Monagas M, Pozo-Bayón MA, Martín-Alvárez PJ, Bartolomé B, Del Campo R, Avila M, Martínez-Cuesta MC, Pelaez C, Moreno-Arribas MV (2010) Perspectives of the potential implications of wine polyphenols on human oral and gut microbiota. Trends Food Sci Technol 21:332−344

Russell WR, Duncan SH, Scobbie L, Duncan G, Cantlay L, Calder AG, Anderson SE, Flint HJ (2013) Major phenylpropanoid-derived metabolites in the human gut can arise from microbial fermentation of protein. Mol Nutr Food Res 57:523–535

Sánchez-Patán F, Monagas M, Moreno-Arribas MV, Bartolomé B (2011) Determination of microbial phenolic acids in human faeces by UPLC−ESI-TQ MS. J Agric Food Chem 59:2241−2247

Sánchez-Patán F, Cueva C, Monagas M, Walton GE, Gibson GR, Quintanilla-López JE, Lebrón-Aguilar R, Martín-Álvarez PJ, Moreno-

Arribas MV, Bartolomé B (2012a) *In vitro* fermentation of a red wine extract by human gut microbiota: Changes in microbial groups and formation of phenolic metabolites. J Agric Food Chem 60:2136−2147

Sánchez-Patán F, Tabasco R, Monagas M, Requena T, Peláez C, Moreno-Arribas MV, Bartolomé B (2012b) Capability of *Lactobacillus plantarum* IFPL935 to catabolize flavan-3-ol compounds and complex phenolic extracts. J Agric Food Chem 60:7142−7151

Selma MV, Espín JC, Tomás-Barberán FA (2009) Interaction between phenolics and gut microbiota: Role in human health. J Agric Food Chem 57:6485−6501

Stevenson DE, Hurst RD (2007) Polyphenolic phytochemicals--just antioxidants or much more? Cell Mol Life Sci 64:2900–2916

Tabasco R, Sánchez-Patán F, Monagas M, Bartolomé B, Moreno-Arribas MV, Peláez C, Requena T (2011) Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: Resistance and metabolism. Food Microbiol 28:1345−1352

Tomás-Barberán FA, Andrés-Lacueva C (2012) Polyphenols and health: current state and progress. J Agric. Food Chem. 60, 8773−8775

Tzounis X, Vulevic J, Kuhnle GC, George T, Leonczak J, Gibson GR, Kwik-Uribe C, Spencer JP (2008) Flavonol monomerinduced changes to the human faecal microflora. Br J Nutr 99:782−792

Tzounis X, Rodriguez-Mateos A, Vulevic J, Gibson GR, Kwik-Uribe C, Spencer JPE (2011) Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. Am J Clin Nutr 93: 62-72

Van de Wiele T, Boon N, Possemiers S, Jacobs H, Verstraete W (2007) Inulin-type fructans of longer degree of polymerization exert more pronounced *in vitro* prebiotic effects. J Appl Microbiol 102:452–460

Van den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gérard P, Rabot S, Bruneau A, El Aidy S, Derrien M, Zoetendal E, Kleerebezem M, Smidt H, Van de Wiele T (2010) Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and *Clostridium* cluster IX. Appl Environ Microbiol 76:5237−5246

Van den Abbeele P, Venema K, Van de Wiele T, Verstraete W, Possemiers S (2013) Different human gut models reveal the distinct fermentation patterns of arabinoxylan versus inulin. J Agric Food Chem 61:9819–9827

Van Dorsten FA, Peters S, Gross G, Gómez-Roldán V, Klinkenberg M, De Vos RC, Vaughan EE, Van Duynhoven JP, Possemiers S, Van de Wiele T, Jacobs DM (2012) Gut microbial metabolism of polyphenols from black tea and red wine/grape juice is source-specific and colon-region dependent. J Agric Food Chem 60:11331−11342

Van Duynhoven J, Vaughan EE, Jacobs DM, Kemperman RA, Van Velzen EJ, Gross G, Roger LC, Possemiers S, Smilde AK, Doré J, Westerhuis JA, Van de Wiele T (2011) Metabolic fate of polyphenols in the human superorganism. Proc Natl Acad Sci USA 108:4531−4538

Wang LQ, Meselhy MR, Li Y, Nakamura N, Min BS, Qin GW, Hattori M (2001) The heterocyclic ring fission and dehydroxylation of catechins and related compounds by *Eubacterium* sp. strain SDG-2, a human intestinal bacterium. Chem Pharm Bull 49:1640−1643

Ward NC, Croft KD, Puddey IB, Hodgson JM (2004) Supplementation with grape seed polyphenols results in increased urinary

excretion of 3-hydroxyphenylpropionic acid, an important metabolite of proanthocyanidins in humans. J Agric Food Chem 52:5545–5549

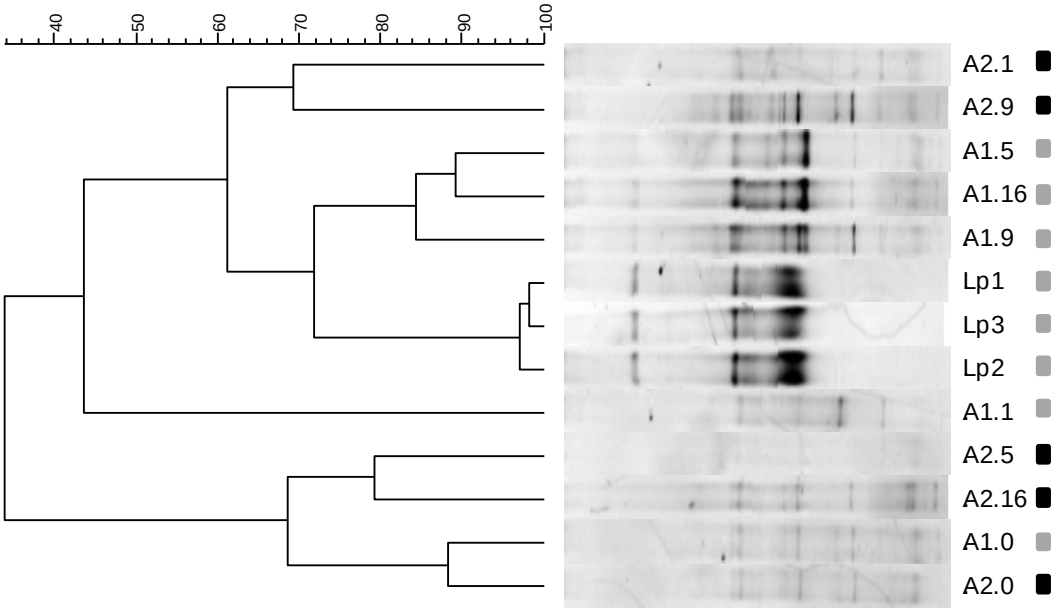
Waterhouse AL (2002) Wine phenolics. Ann NY Acad Sci 957:21−36

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD (2011) Linking long-term dietary patterns with gut microbial enterotypes. Science 334:105–108

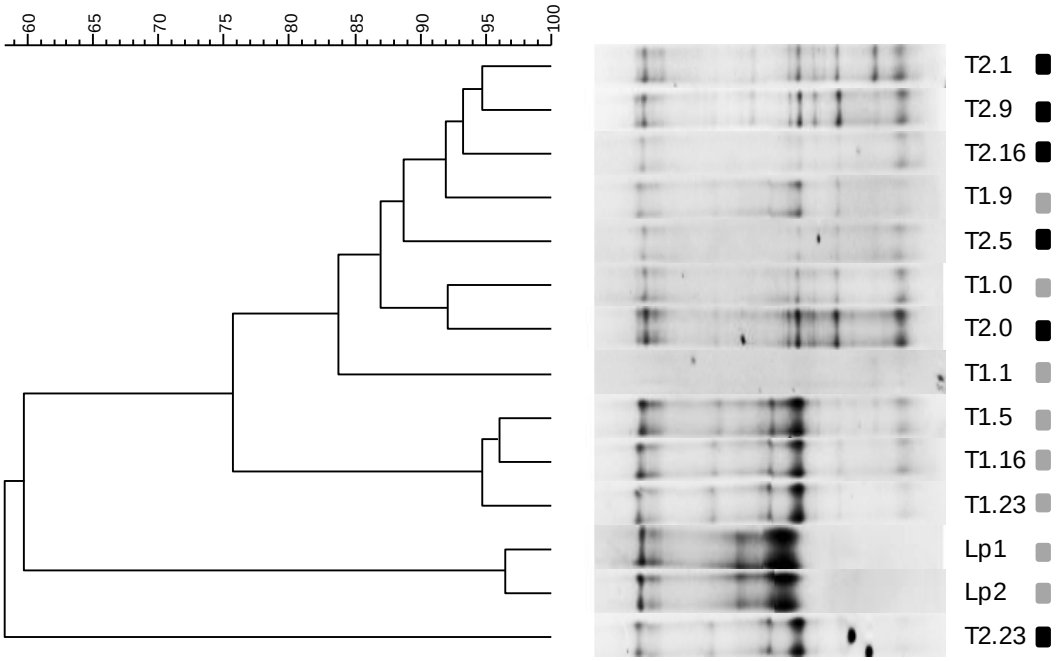
Xia EQ, Deng GF, Guo YJ, Li HB (2010) Biological activities of polyphenols from grapes. Int J Mol Sci 11:622–646

V.7 SUPPLEMENTAL MATERIAL

Ascending Colon



Transverse colon



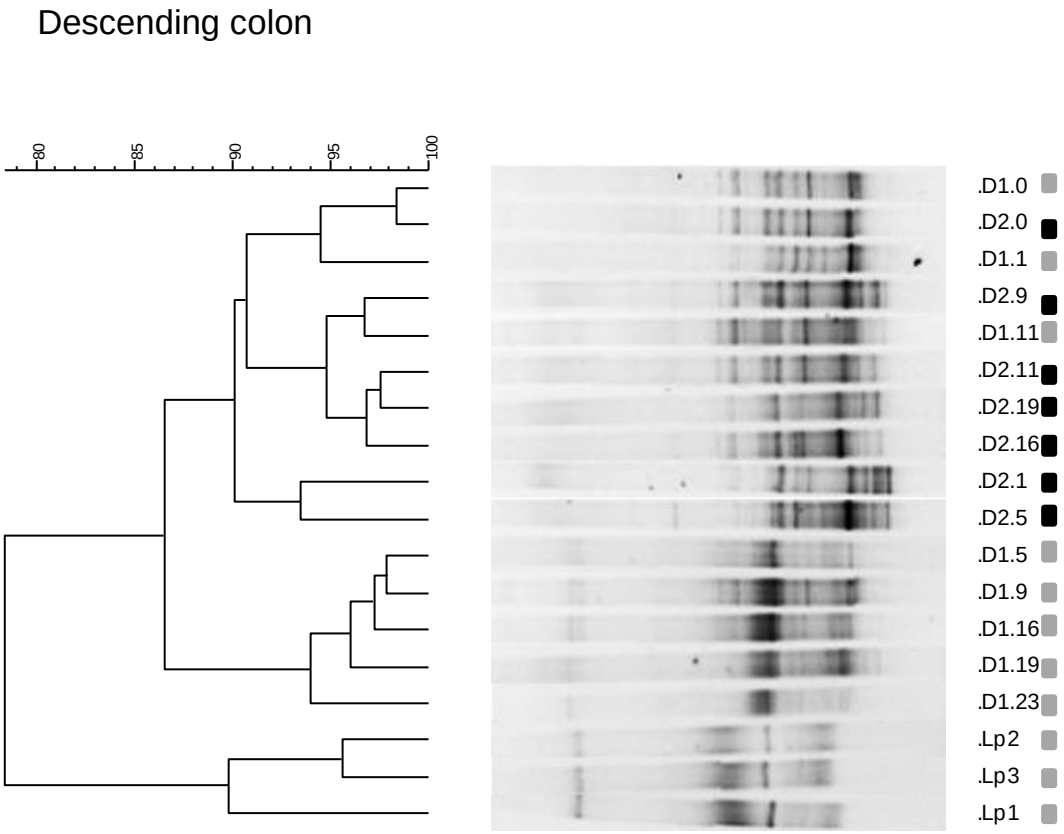


Figure S1. Clustering tree of the Lactobacillus DGGE-profiles in the ascending, transverse and descending colon of the Twin- SHIME during the treatment (days 0, 1, 5, 9) with the red wine phenolic extract (black lanes) and the extract supplemented with L. plantarum IFPL935 (grey lanes) and the wash-out period (days 16, 19, 23). Lp, DNA from L. plantarum IFPL935.

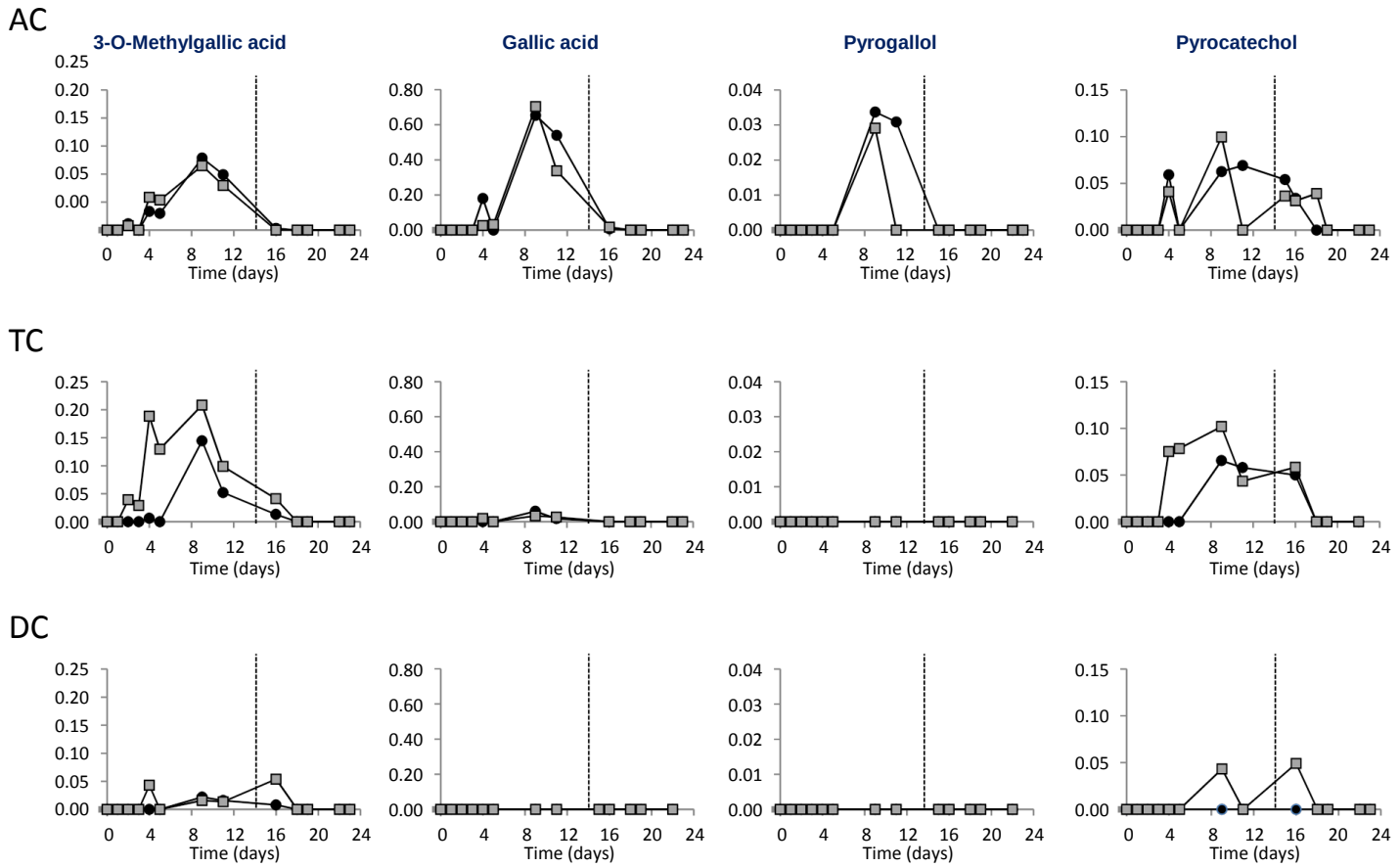


Figure S2. Changes in concentration (µg/ml) of galloylated compounds and their metabolites in the ascending (AC), transverse (TC) and descending colon (DC) of the Twin-SHIME during the treatment with the red wine phenolic extract (grey symbols) and the extract supplemented with L. plantarum IFPL935 (black symbols) and the wash-out period. The dotted line indicates the end of treatment

Table S1. Concentration (mean values in µg/mL) of the phenolic metabolites measured.

Compound	Colonic vessel	Treatment	Intervention period				
			Day 0 (steady state)	Day 1	Week 1	Week 2	Week 3 (wash out)
Benzoic acids							
3,4,5-triOH-	AC	Provin+Lp935	0.00	0.00	0.05	0.60	0.00
		Provin	0.00	0.00	0.01	0.52	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.04	0.00
		Provin	0.00	0.00	0.00	0.03	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
4,5-diOH-3-OCH_3-	AC	Provin+Lp935	0.00	0.00	0.02	0.11	0.00
		Provin	0.00	0.00	0.03	0.10	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.10	0.00
		Provin	0.00	0.00	0.10	0.15	0.01
	DC	Provin+Lp935	0.00	0.00	0.00	0.02	0.00
		Provin	0.00	0.00	0.01	0.01	0.01
4-OH-3,5-diOCH_3-	AC	Provin+Lp935	0.00	0.00	0.10	0.04	0.01
		Provin	0.00	0.00	0.06	0.04	0.00
	TC	Provin+Lp935	0.00	0.00	0.13	0.08	0.02
		Provin	0.00	0.00	0.13	0.18	0.01
	DC	Provin+Lp935	0.00	0.00	0.07	0.09	0.02
		Provin	0.00	0.00	0.06	0.07	0.02
3,4-diOH-	AC	Provin+Lp935	0.04	0.00	0.08	0.15	0.04
		Provin	0.04	0.00	0.08	0.14	0.03
	TC	Provin+Lp935	0.01	0.00	0.03	0.10	0.01
		Provin	0.02	0.00	0.12	0.16	0.02
	DC	Provin+Lp935	0.01	0.00	0.02	0.04	0.02
		Provin	0.01	0.00	0.01	0.03	0.01
4-OH-3-OCH_3-	AC	Provin+Lp935	0.05	0.00	0.10	0.08	0.04
		Provin	0.06	0.00	0.10	0.07	0.04
	TC	Provin+Lp935	0.07	0.01	0.08	0.09	0.05
		Provin	0.07	0.03	0.08	0.11	0.04
	DC	Provin+Lp935	0.07	0.04	0.06	0.06	0.04
		Provin	0.06	0.03	0.04	0.06	0.04
4-OH-	AC	Provin+Lp935	0.06	0.06	0.07	0.06	0.06
		Provin	0.06	0.05	0.06	0.06	0.04
	TC	Provin+Lp935	0.10	0.07	0.10	0.06	0.05
		Provin	0.10	0.06	0.07	0.07	0.06
	DC	Provin+Lp935	0.09	0.06	0.09	0.03	0.06
		Provin	0.09	0.00	0.04	0.03	0.02
3-OH-	AC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
	TC	Provin+Lp935	0.01	0.00	0.00	0.00	0.01
		Provin	0.01	0.00	0.00	0.01	0.02
	DC	Provin+Lp935	0.01	0.00	0.00	0.00	0.01
		Provin	0.01	0.00	0.00	0.00	0.01

2-OH-	AC	Provin+Lp935	0.02	0.02	0.02	0.04	0.02
		Provin	0.02	0.03	0.03	0.03	0.02
	TC	Provin+Lp935	0.02	0.02	0.03	0.05	0.03
		Provin	0.02	0.03	0.04	0.04	0.03
	DC	Provin+Lp935	0.02	0.02	0.04	0.05	0.03
		Provin	0.02	0.03	0.04	0.06	0.05
Benzoic	AC	Provin+Lp935	1.50	1.12	0.72	0.77	0.22
		Provin	1.53	0.80	0.61	0.27	0.20
	TC	Provin+Lp935	1.61	1.04	0.67	0.27	0.21
		Provin	1.56	1.22	1.12	0.23	0.21
	DC	Provin+Lp935	1.60	1.28	0.84	0.29	0.31
		Provin	1.24	1.49	0.95	0.24	0.25
Phenols							
2,3-diOH-	AC	Provin+Lp935	0.00	0.00	0.00	0.03	0.00
		Provin	0.00	0.00	0.00	0.01	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
2-OH-	AC	Provin+Lp935	0.00	0.00	0.01	0.07	0.01
		Provin	0.00	0.00	0.01	0.05	0.02
	TC	Provin+Lp935	0.00	0.00	0.00	0.06	0.01
		Provin	0.00	0.00	0.04	0.07	0.01
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
4-(2-OHethyl)-	AC	Provin+Lp935	0.00	0.00	0.39	0.00	0.00
		Provin	0.00	0.00	1.33	2.89	0.15
	TC	Provin+Lp935	0.00	0.00	1.66	4.99	0.76
		Provin	0.00	0.00	2.27	5.34	0.39
	DC	Provin+Lp935	0.00	0.00	1.78	5.25	1.25
		Provin	0.00	0.00	0.00	0.00	0.48
Phenylacetic acids							
3,4-diOH-	AC	Provin+Lp935	0.05	0.00	0.01	0.06	0.03
		Provin	0.07	0.00	0.03	0.83	0.07
	TC	Provin+Lp935	0.05	0.00	0.00	0.26	0.04
		Provin	0.06	0.00	0.09	1.19	0.14
	DC	Provin+Lp935	0.05	0.06	0.02	0.00	0.01
		Provin	0.05	0.05	0.00	0.06	0.05
4-OH-	AC	Provin+Lp935	1.42	1.40	0.71	3.46	2.18
		Provin	4.90	1.01	1.00	2.29	3.06
	TC	Provin+Lp935	1.66	0.39	0.82	0.97	1.27
		Provin	1.85	0.95	2.94	2.04	2.15
	DC	Provin+Lp935	0.93	0.26	0.45	0.66	0.74
		Provin	0.34	0.13	0.40	0.27	0.63
3-OH-	AC	Provin+Lp935	0.08	0.00	0.01	0.07	0.08
		Provin	0.19	0.05	0.02	0.07	0.08
	TC	Provin+Lp935	0.24	0.12	0.06	0.15	0.17
		Provin	0.24	0.12	0.10	0.15	0.19

	DC	Provin+Lp935	0.20	0.14	0.06	0.41	0.29
		Provin	0.22	0.12	0.10	0.29	0.30
Phenylacetic	AC	Provin+Lp935	8.9	4.0	2.6	17.7	14.2
		Provin	14.6	4.9	3.7	16.3	14.6
	TC	Provin+Lp935	18.0	12.0	10.6	24.1	26.4
		Provin	19.6	11.7	12.8	24.7	26.8
	DC	Provin+Lp935	19.5	16.5	11.2	24.7	26.2
		Provin	20.6	13.2	9.6	24.4	24.6
Phenypropionic acids							
3,4-diOH-	AC	Provin+Lp935	0.00	0.00	0.06	0.61	0.02
		Provin	0.00	0.00	0.05	0.57	0.02
	TC	Provin+Lp935	0.00	0.00	0.00	0.33	0.03
		Provin	0.00	0.00	0.06	0.39	0.05
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.02	0.02	0.01
4-OH-	AC	Provin+Lp935	0.0	0.0	0.3	0.8	0.0
		Provin	0.0	0.0	0.3	0.9	0.1
	TC	Provin+Lp935	12.1	5.8	4.1	9.0	6.7
		Provin	3.0	0.8	1.9	6.6	5.9
	DC	Provin+Lp935	12.1	9.2	3.8	9.0	6.4
		Provin	6.4	0.0	0.9	5.9	6.1
3-OH-	AC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.01
	TC	Provin+Lp935	0.02	0.01	0.01	0.00	0.01
		Provin	0.01	0.01	0.00	0.00	0.00
	DC	Provin+Lp935	0.07	0.17	0.12	3.78	1.59
		Provin	0.01	0.02	0.09	1.48	1.49
Phenylpropionic	AC	Provin+Lp935	0.00	0.00	0.00	0.00	0.04
		Provin	0.00	0.00	0.00	0.00	0.06
	TC	Provin+Lp935	0.00	0.00	0.45	0.00	0.04
		Provin	0.00	0.00	0.00	0.00	0.02
	DC	Provin+Lp935	0.00	0.00	0.46	0.00	0.07
		Provin	0.00	0.00	0.01	0.00	0.00
4-OH-5-( )-valeric acids							
(3,4-diOHphenyl)	AC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.04	0.00
		Provin	0.00	0.00	0.01	0.06	0.01
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.01	0.01	0.00
(phenyl)	AC	Provin+Lp935	0.0	0.0	0.0	0.0	0.0
		Provin	0.0	0.0	0.0	0.0	0.0
	TC	Provin+Lp935	0.0	0.0	0.4	2.4	1.3
		Provin	0.0	0.0	0.0	4.3	1.5
	DC	Provin+Lp935	0.0	0.0	0.1	46.1	13.5
		Provin	0.0	0.0	0.0	49.5	13.7
5-( )-γ-valerolactones							
-(3,4-diOHphenyl)-	AC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00

	TC	Provin+Lp935	0.00	0.00	0.00	0.03	0.00
		Provin	0.00	0.00	0.01	0.07	0.01
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
Cinnamic acids							
3,4-diOH-	AC	Provin+Lp935	0.00	0.00	0.03	0.00	0.00
		Provin	0.00	0.00	0.02	0.00	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.01	0.00	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
4-OH-3-OCH_3-	AC	Provin+Lp935	0.00	0.02	0.01	0.00	0.00
		Provin	0.00	0.02	0.02	0.00	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
4-OH-	AC	Provin+Lp935	0.01	0.00	0.06	0.01	0.00
		Provin	0.00	0.01	0.05	0.02	0.00
	TC	Provin+Lp935	0.08	0.02	0.04	0.03	0.00
		Provin	0.03	0.01	0.05	0.03	0.00
	DC	Provin+Lp935	0.07	0.06	0.03	0.02	0.01
		Provin	0.06	0.01	0.01	0.04	0.02

Table S2. Concentration (mean values in µg/mL) of the phenolic compounds from the wine extract.

Compound	Colonic vessel	Treatment	Intervention period				
			Day 0 (steady state)	Day 1	Week 1	Week 2	Week 3 (wash out)
Flavan-3-ols							
(+)-Catechin	AC	Provin+Lp935	0.00	0.00	0.11	0.47	0.01
		Provin	0.00	0.00	0.11	0.58	0.02
	TC	Provin+Lp935	0.00	0.00	0.01	0.06	0.00
		Provin	0.00	0.00	0.28	0.02	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.01	0.00
		Provin	0.00	0.00	0.01	0.01	0.00
(-)-Epicatechin	AC	Provin+Lp935	0.00	0.00	0.03	0.26	0.00
		Provin	0.00	0.00	0.03	0.34	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.11	0.01	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
Procyanidin B1	AC	Provin+Lp935	0.00	0.00	0.03	0.16	0.00
		Provin	0.00	0.00	0.02	0.06	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.03	0.00
		Provin	0.00	0.00	0.11	0.00	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.05	0.00	0.00
Procyanidin B2	AC	Provin+Lp935	0.00	0.00	0.03	0.15	0.00
		Provin	0.00	0.00	0.02	0.04	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.07	0.00
		Provin	0.00	0.00	0.10	0.01	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.02	0.00	0.00
Procyanidin B3	AC	Provin+Lp935	0.00	0.00	0.00	0.06	0.00
		Provin	0.00	0.00	0.01	0.07	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.04	0.06	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.01	0.00	0.00
Procyanidin B4	AC	Provin+Lp935	0.00	0.00	0.00	0.10	0.00
		Provin	0.00	0.00	0.01	0.08	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.08	0.00
		Provin	0.00	0.00	0.03	0.03	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.02	0.00	0.00
Anthocyanins							
Malvidin-3-glu	AC	Provin+Lp935	0.00	0.00	0.01	0.02	0.00
		Provin	0.00	0.00	0.01	0.03	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
Flavonols							

Quercetin	AC	Provin+Lp935	0.00	0.00	0.06	0.24	0.05
		Provin	0.00	0.00	0.05	0.00	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
	DC	Provin+Lp935	0.00	0.00	0.00	0.00	0.00
		Provin	0.00	0.00	0.00	0.00	0.00
Stilbenes							
Resveratrol	AC	Provin+Lp935	0.00	0.00	0.03	0.00	0.00
		Provin	0.00	0.00	0.04	0.03	0.00
	TC	Provin+Lp935	0.00	0.00	0.00	0.03	0.00
		Provin	0.00	0.00	0.06	0.05	0.00
	DC	Provin+Lp935	0.00	0.00	0.01	0.02	0.00
		Provin	0.00	0.00	0.03	0.03	0.00

VI. STABILITY OF SALIVA MICROBIOTA DURING MODERATE CONSUMPTION OF RED WINE

Manuscript in preparation

VI.1 ABSTRACT

This study has evaluated the effect of regular and moderate red wine consumption on the diversity and occurrence of different groups of bacteria that are representative in human saliva. Saliva was obtained from individuals before and after consumption of red wine (250 mL/day) during 4 weeks. The evolution and composition of the microbial community in saliva was evaluated by PCR-DGGE and quantitative PCR. In general, the inter-individual variability observed in the PCR-DGGE band patterns was higher than the differences observed after the 4-weeks period of red wine intake. *Bifidobacterium* dentium, *Bifidobacterium* spp. and *Alloscardovia omnicolens* were the most representative bifidobacterial species, whereas the *Streptococcus mitis*-*Streptococcus oralis* group predominated within *Streptococcus*. This genus was the most numerous of the bacterial groups assayed, reaching average counts above 8 log copy numbers/mL. On the other hand, the lowest counts were recorded for *Actinomyces*, *Fusobacterium*, *Haemophilus*, *Neisseria* and *Veillonella*, which showed average values of 5 log copy numbers/mL. The results showed no significant differences ($P>0.5$) in bacterial counts after the period of red wine intake. In conclusion, the overall diversity and stability of representative bacterial groups of the human saliva is not disturbed due to regular-moderate red wine consumption.

VI.2 INTRODUCTION

Regular intake of polyphenol-rich beverages and foods has demonstrated to exert beneficial effects in human health, such as decreased incidence of cardiovascular disease, cancer and protection against neurodegenerative diseases, among others [1-3]. Nevertheless, the beneficial effects of polyphenols seem to be more linked to microbial phenolic metabolites produced in the human gut than to the original forms present in food [4]. Accordingly, regular and moderate intake of red wine (a characteristic polyphenol-rich beverage) has demonstrated to exert modulating effects in the human gut microbiota [5-7]. It is reasonable to presume that besides their effect on gut microbiota, these polyphenols can exert an effect on the overall oral cavity microbiota (saliva and gingival margins). This is worth considering that diversity of the microbial populations in the oral cavity is even larger than in the gut or the skin, harboring viruses, archea, protozoa, fungi and over 700 species of bacteria [8, 9]. Similarly to human gut or skin microbiome, the oral microbial community is an interacting ecosystem with the host that helps to maintain the health status, although certain ecological shifts allow pathogens to establish and cause disease [10, 11]. Despite its relevance to human health, little information is currently available on the effect, if any, of daily habits as regular red wine consumption on both saliva and gingival dental microbiota.

The antimicrobial effects of the polyphenols present in red wine and grape seed extracts against microorganisms responsible for periodontitis and dental caries have been mainly studied by incubating polyphenols with pure strains [12-15]. Signoretto et al. [16] evaluated the microbial composition of supragingival and subgingival plaque in 75 adult volunteers that had been drinking 400 ml red wine daily for at least two previous years. It was observed a lower microbial diversity in the plaque samples of regular wine drinkers compared with water drinkers. This

microbial modulating effect was speculatively attributed to the antimicrobial, antiadhesive, and antiplaque activities of the polyphenols contained in wine. In a review targeting bacterial adhesion to different susbstrates, the same authors considered the polyphenol-rich foods as a potential alternative strategy to antibiotic therapy in order to avoid caries and gingivitis/periodontitis [17].

Concerning saliva, the effect of consumption of red wine on the antioxidant status of this human fluid has been recently investigated [18], but information on the effect of red wine in the microbial community of saliva is still scarce. Saliva is crucial to oral cavity health, containing a characteristic bacterial community that helps maintaining homeostasis of the mouth ecosystem and it is considered useful in prognosis of several systemic and oral dysfunctions [10, 19]. Oral health is characterized by an ecologically balanced and diverse microbiome and the analysis of plaque and saliva in healthy adults has demonstrated larger microbial biodiversity than in people suffering symptoms of caries or periodontitis [10]. It is therefore essential to health the maintenance of oral cavity homeostasis through a well-balanced and stable oral microbiome in which saliva plays a key role.

Recent metagenomic studies have revealed that *Streptococcus*, *Veillonella*, *Neisseira*, *Prevotella* and *Fusobacterium* are among the most predominant microorganisms in the saliva of healthy individuals [20–22]. Nevertheless, under various circumstances, oral homeostasis is lost and certain species from these and other genera, such as *Haemophylus*, *Lactobacillus* and *Porphyromonas*, turn out to be opportunistic and can represent a potential threat for both oral and respiratory tract health status [10, 23-24].

The purpose of this work has been to study if the regular and moderate consumption of red wine during four weeks can affect somehow the diversity and occurrence of different groups and genera of bacteria

that are representative in human saliva and hence, the local homeostasis needed to keep oral health.

VI.3 MATERIALS AND METHODS

VI.3.1 Wine intervention study and sampling

Saliva samples were obtained during the human intervention study with moderate intake of red wine described by [25]. Briefly, 22 healthy non-smokers volunteers (age range 20−48 years), included in the wine intervention trial (14 individuals) and in the control group (8 volunteers), provided saliva samples at the beginning of the study and after 4 weeks. The intervention group consumed daily 250 mL of red wine (equivalent to a daily dose of approx. 450 mg of total polyphenols) and individuals were asked to not consume any other alcoholic beverages and to follow a low-polyphenols diet. The control group followed the same pattern regarding restrictions for alcoholic beverages and polyphenols. Previously to the provision of the first sample, both groups had followed an initial washout period of 2 weeks (baseline) with the same restrictions in alcohol and polyphenols. Unstimulated saliva was collected at morning from the subjects before brushing their teeth and consuming foods or beverages. Samples were placed in tightly covered sterile bottles and delivered to the laboratory in less than 2 h for treatment and analyses.

VI.3.2 Sample preparation and DNA extraction

Saliva samples were centrifuged at 10000 ×g, 15 min at 4 °C, and pellets were stored at -80 °C with 40% glycerol. For DNA extraction, samples were thawed, mixed with 100 µL sodium phosphate buffer, pH 7.0, and 100 mg of glass beads (150−212 µm diameter). Cells were mechanically lysed by using a FastPrep equipment (BIO 101, Savant Instruments, Holbrook, NY) for 45 s (three times) at a machine speed

setting of 5.5 m/s. The aqueous phase containing genomic DNA was separated by centrifugation, 10000 ×g for 10 min at 4 °C, and DNA was extracted using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany).

VI.3.3 PCR-DGGE

The diversity of the bacterial communities in the saliva samples and the effect of the red wine consumption were assessed by PCR-DGGE and using the primers and annealing temperatures described in Table VI.1. Samples were tested for total bacteria, *Streptococcus*, *Veillonella*, *Neisseria*, *Lactobacillus*, *Bifidobacterium* and the group *Prevotella*/*Porphyromonas*/*Bacteroides*. For *Lactobacillus* and *Bifidobacterium*, a nested PCR step was included that was carried out as described by Heilig et al. [26] and Satokari et al. [27], respectively. DGGE was performed with a DCode system (Bio-Rad) using a 9%polyacrylamide gel and a genus-specific gradient (Table VI.1) of 7 M urea and 40 % formamide. The obtained band patterns were analyzed using InfoQuest FP software version 5.1 (Bio-Rad). Selected DGGE bands were sequenced after excision from the gel and PCR reamplificated with each specific primer set.

VI.3.4 Quantitative PCR (qPCR)

Total bacteria and different groups and genera of bacteria that are representative in human saliva (Table VI.1) were quantified by qPCR using SYBR green methodology (Kappa Biosystems, Woburn, MA, USA) with the IQ5 Multicolor Real-Time PCR Detection System and data analyses (Bio-Rad Laboratories Inc., Hercules, USA). DNA from *Escherichia coli* DH5α, *Lactobacillus plantarum* IFPL935, *Bifidobacterium lactis* Bb12 and *Streptococcus* thermophilus ATCC 1987 was used for quantification of total bacteria, *Lactobacillus*, *Bifidobacterium* and

Streptococcus, respectively. For the rest of the groups analyzed (*Actinomyces*, *Fusobacterium*, *Haemophilus*, *Neisseria*, *Veillonella* and *Prevotella*), samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega, Madison, WI, USA), as described previously Barroso et al. [28].

VI.3.5 Statistical analysis

Mean values and standard deviations were calculated on the basis of the qPCR values for the different bacterial groups before and after the 4-week study period, for both the control and intervention groups. The t-test for dependent samples and its corresponding nonparametric Wilcoxon matched-pairs test was used to evaluate differences in the counts of bacterial groups before and after the 4-week period. Statistical analyses were carried out using the IBM SPSS Statistics 21 package (SPSS Inc., Chicago, IL, USA).

Table VI.1. Primer sets used in this study for PCR-DGGE and quantitative PCR.

Target group	Primer sequence 5'-3'	Annealing (°C)	Gradient (%)	reference
PCR-DGGE				
Total bacteria	F: AACGCGAAGAACCTTAC+GC	56	30-60	36
	R: CGGTGTGTACAAGACCC			
Lactobacillus	F: GGAAACAGGTGCTAATACCG	56	30-50	26
	R: ATCGTATTACCGCGGCTGCTGGCAC+GC			
	F: GTTTGATCCTGGCTCAG	66	nested	26
	R: CACCGCTACACATGGAG			
Bifidobacterium	F: GGGTGGTAATGCCGGATG	62	40-55	27
	R: GCCACCGTTACACCGGGAA			
	F: CGGGTGCTICCCACTTTCATG	57	nested	27
	R: GATTCTGGCTCAGGATGAACG			
Streptococcus	F: AGATGGACCTGCGTTGT+GC	55	25-50	37
	R: GTGAACTTTCCACTCTCACAC			
Veillonella	F: A(C/T)CAACCTGCCCTTCAGA	62	40-70	38
	R: CGTCCCGATTAACAGAGCTT+GC			
Prevotella-Porphiromonas-	F: GGTGTCGGCTTAAGTGCCAT+GC	68	40-58	38
Bacterioides	R: CGGA(C/T)GTAAGGGCCGTGC			
Neisseria	F: CTGGCGCGGTATGGTCGGTT	55	30-70	39
	R: GCCGACGTTGGAAGTGGTAAAG+GC			
Quantitative PCR				
Total bacteria	F: CGGTGAATACGTTC(C/T)CGG	59		40
	R: CGGTGTGTACAAGACCC			
Actinomyces	F: CTCCTACGGGAGGCAGCAG	60		41
	R: CACCCACAAACGAGGCAG			
Bifidobacterium	F: CTCCTGGAAACGGGTGG	55		42
	R: GGTGTTCTTCCCGATATCTACA			
Fusobacterium	F: C(A/T)AACGCGATAAGTAATC	54		38
	R: TGGTAACATACGA(A/T)AGGG			
Haemophilus	F: GGAGTGGGTTGTACCAGAAGTAGAT	55		43
	R: AGGAGGTGATCCAACCGC			
Lactobacillus	F: TGGAAACAG(A/G)TGCTAATACCG	62		40
	R: GTCCATTGTGGAAGATTCCC			
Neisseria	F: CTGGCGCGGTATGGTCGGTT	55		39
	R: GCCGACGTTGGAAGTGGTAAAG			
Prevotella	F: CAC(A/G)GTAAACGATGGATGCC	55		43
	R: GGTCGGGTTGCAGACC			
Streptococcus	F: AGATGGACCTGCGTTGT	55		37
	R: GTGAACTTTCCACTCTCACAC			
Veillonella	F: A(C/T)CAACCTGCCCTTCAGA	62		38
	R: CGTCCCGATTAACAGAGCTT			

GC clamp at 5': CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC

VI.4 RESULTS AND DISCUSSION

The potential changes in the diversity of bacterial groups after daily consumption of 250 mL of red wine during four weeks were analyzed by PCR-DGGE. The usefulness of applying DGGE to monitor temporal and developmental changes in oral bacterial communities from saliva and dental plaque has been demonstrated by Rasiah et al [29]. Results using primers targeting total bacteria showed no apparent changes in the band patterns due to red wine intake (Fig. S1, supplementary material). In general, the inter-individual variability in the band patterns was higher than the differences observed after the 4-weeks period of red wine intake.

Considering the observed differences in sensibility of specific oral microbial groups to wine and grape seed polyphenols [15, 30], we evaluated the PCR-DGGE band profiles of representative oral bacterial groups before and after red wine intake. As an example, Fig. VI.1 shows the *Streptococcus* and *Bifidobacterium* fingerprints of eight individuals before and after red wine intake. Results distinguished in the *Bifidobacterium* PCR-DGGE patterns three main bands that, after nucleotide sequencing, were identified to correspond with *Bifidobacterium* dentium, *Bifidobacterium* spp. and *Alloscardovia omnicolens* (Fig. VI.1A). This last species has recently been described to belong to the Bifidobacteriaceae family [31] and to be associated with human saliva [32]. Again, more differences in the *Bifidobacterium* fingerprints were observed between individuals than over the period of red wine intake in each individual. Similar results were observed following the specific PCR-DGGE analysis of *Streptococcus* (Fig. VI.1B). In this case, the predominant band was sequenced and identified as belonging to the *S. mitis-S. oralis* group. *S. mitis* is the most common bacterium isolated from the oral cavity [22]. Regarding the rest of bacterial genera examined, no consistent fingerprint difference associated to red wine intake was either

observed for *Lactobacillus*, *Veillonella*, *Neisseria* or *Prevotella-Porphyromonas-Bacteroides* group (results not shown).

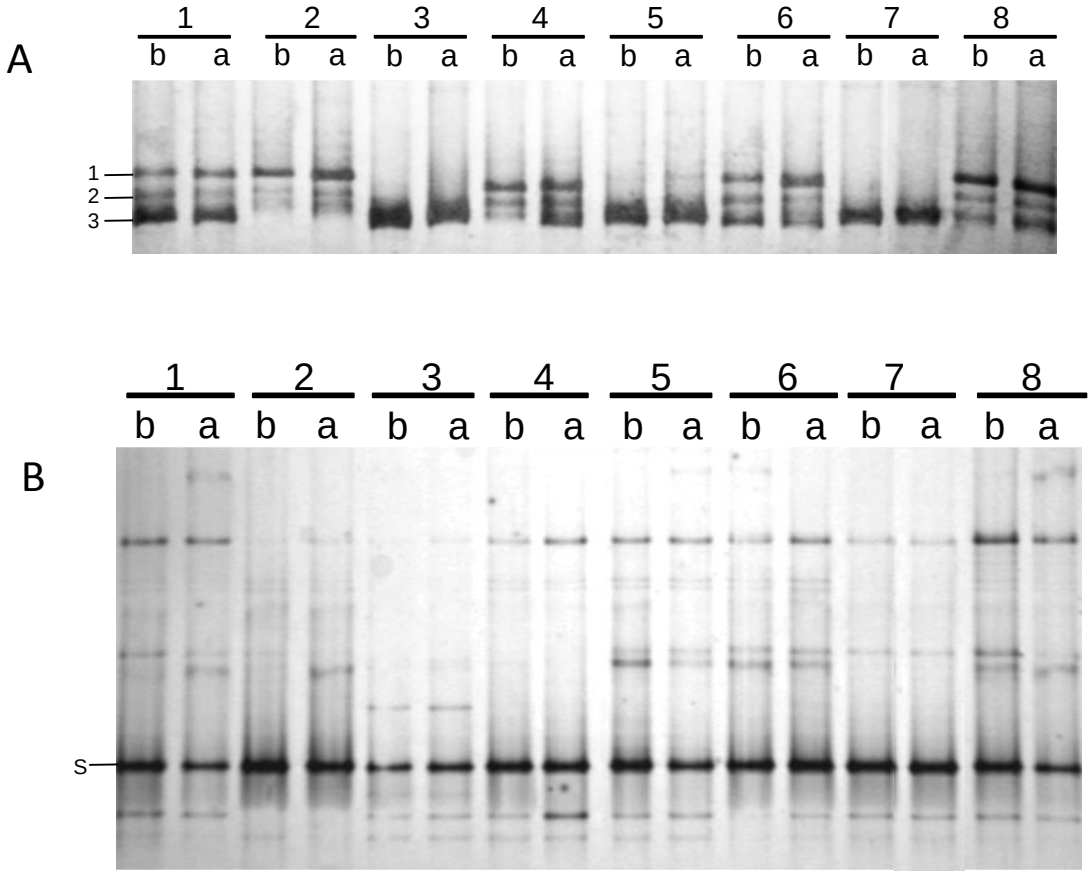


Figure VI.1. PCR-DGGE band profiles from eight individuals (out of fourteen) from the intervention group before (b) and after (a) red wine intake, obtained from saliva DNA and specific primers for *Bifidobacterium* (A) and *Streptococcus* (B). Bands 1: *Bifidobacterium* dentium, 2: *Bifidobacterium* spp., 3: *Alloscardovia omnicolens*, S: group *Streptococcus mitis-Streptococcus oralis.*

A complementary approach to evaluate the effect of red wine intake was the evaluation by qPCR of the potential quantitative changes of representative bacterial groups present in the oral cavity (Table VI.2). Total bacterial counts in saliva showed an average of 8 log copy numbers/mL in all individuals before and after the timeframe of the study. These figures are within the range of 10^7 to 10^9 bacteria per mL described for human salivary microbiota [33]. *Streptococcus* were the most numerous of the bacterial groups assayed since all samples showed average counts above 8 log copy numbers/mL. Average counts for *Lactobacillus* and *Prevotella* were about 7 log copy numbers/mL (Table

VI.2). On the other hand, among all the bacterial groups analyzed, the lowest counts were recorded for *Actinomyces* that did not reach values higher than 5 log copy numbers/mL, which in turn was the average value recorded for *Fusobacterium*, *Haemophilus*, *Neisseria* and *Veillonella*. The results are in agreement with metagenomic studies which reveal that the oral cavity is generally dominated by *Streptococcus* species [8, 20].

The t-test for dependent samples and its corresponding nonparametric Wilcoxon matched-pairs test showed no significant differences between samples before and after the 4-week study period, with P values higher than 0.1 in all cases (Table VI.2), indicating that no differences in bacterial counts could be attributable to the red wine intake. Due to the high variations in counts between individuals, t-test analyses were also performed distinguishing subjects grouped by initial counts being either higher or lower than the average values. Again, the results indicated no statistical differences in counts of any of the bacterial groups assayed.

The stability of numbers and composition of the saliva microbiota over the 4-week period of red wine intake shown in this work is in agreement with metagenomic studies indicating high levels of variability of human-associated microbial communities among individuals and minimal variations over time within a given human body habitat, such as the oral cavity [8]. This oral stability is kept despite age, gender or daily ambient fluctuations including diet and mechanical forces from brushing and mastication. Longitudinal surveys of salivary bacterial communities in twins from adolescence to adulthood have shown a long term stable microbiome in the saliva, more related to cohabitation of individuals than weight class or gender [22]. In this regard, one element to be considered for stable oral microbiota is the short time of exposition of this community to polyphenols in comparison with the estimated accumulation of dietary polyphenols in colon [34]. Accordingly, a recent study with 12 individuals

showed that salivary antioxidant capacity did not increase after drinking 125 mL of red wine [18]. Another relevant consideration is resilience of the oral microbiota, which implies the degree to which the microbial community returns to its former state after a disturbance caused for example by a therapeutic intervention [35].

Table VI.2. Quantitative PCR counts (mean ± SD and range values in copy number/mL) for the microbial groups analyzed in saliva from individuals before and after the wine intervention study

Bacteria	Control group (n=8)			Intervention group (n=14)		
	before	after	*P* [a]	before	after	*P* [a]
Total bacteria	8.04 ± 0.56 (7.05-8.57)	7.78 ± 0.38 (7.44-8.56)	0.176	7.98 ± 0.40 (7.47-8.60)	7.80 ± 0.61 (6.65-8.67)	0.133
Actinomyces	4.67 ± 0.52 (3.94-5.29)	4.46 ± 0.39 (4.07-5.13)	0.161	4.89 ± 0.32 (4.43-5.56)	4.77 ± 0.52 (3.88-5.63)	0.279
Bifidobacterium	6.52 ± 0.19 (6.38-6.90)	6.31 ± 0.19 (6.11-6.65)	0.091	7.01 ± 0.44 (6.22-7.60)	6.76 ± 0.45 (6.12-7.57)	0.131
Fusobacterium	5.41 ± 0.89 (4.14-6.93)	5.13 ± 0.57 (4.18-5.85)	0.327	5.52 ± 0.77 (4.37-6.89)	5.60 ± 0.92 (3.98-6.97)	0.600
Haemophilus	5.82 ± 0.81 (4.77-7.33)	5.61 ± 0.55 (4.87-6.38)	0.262	6.09 ± 0.71 (5.16-7.15)	6.23 ± 0.81 (4.86-7.54)	0.463
Lactobacillus	8.03 ± 0.52 (7.12-8.58)	7.70 ± 0.35 (7.34-8.38)	0.116	7.97 ± 0.37 (7.39-8.52)	7.77 ± 0.62 (6.58-8.55)	0.116
Neisseria	5.57 ± 1.08 (4.35-7.58)	5.30 ± 0.79 (4.43-6.70)	0.123	5.85 ± 0.86 (4.89-7.41)	5.93 ± 1.08 (4.10-7.64)	0.675
Prevotella	7.34 ± 0.89 (5.58-8.49)	7.40 ± 0.54 (6.76-8.13)	0.674	7.55 ± 0.39 (7.10-8.24)	7.48 ± 0.78 (5.76-8.58)	0.807
Streptococcus	8.32 ± 0.39 (7.76-8.74)	8.21 ± 0.41 (7.64-8.97)	0.398	8.52 ± 0.51 (7.56-9.13)	8.45 ± 0.63 (7.04-9.10)	0.382
Veillonella	5.57 ± 0.66 (4.35-6.18)	5.23 ± 0.31 (4.70-5.62)	0.123	5.27 ± 0.61 (4.55-6.24)	5.38 ± 0.64 (3.97-6.25)	0.552

[a]P values evaluated with the Student t test for dependent samples and its corresponding nonparametric Wilcoxon matched-pairs test

In conclusion, the results showed that daily consumption of 250 mL red wine during 4 weeks does not change the overall diversity and stability of representative bacterial groups of the human saliva. Microbial equilibrium and homeostasis are maintained over time in the oral cavity and no major perturbations can be observed due to regular-moderate red wine consumption.

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VI.5 REFERENCES

1. Arranz S, Chiva-Blanch G, Valderas-Martínez P, Medina-Remón A, Lamuela-Raventós RM, Estruch R (2012) Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer. Nutrients 4:759–781

2. Bhullar KS, Rupasinghe HP (2013) Polyphenols: multipotent therapeutic agents in neurodegenerative diseases. Oxid Med Cell Longev 2013:891748.

3. Kishimoto Y, Tani M, Kondo K (2013) Pleiotropic preventive effects of dietary polyphenols in cardiovascular diseases. Eur J Clin Nutr 67:532–535

4. Selma MV, Espín JC, Tomás-Barberán FA (2009) Interaction between phenolics and gut microbiota: role in human health. J Agr Food Chem 57:6485–6501

5. Barroso E, Van de Wiele T, Jiménez-Girón A, Muñoz-González I, Martín-Alvárez PJ, Moreno-Arribas MV, Bartolomé B, Peláez C, Martínez-Cuesta MC, Requena T (2014) *Lactobacillus plantarum* IFPL935 impacts colonic metabolism in a simulator of the human gut microbiota during feeding with red wine polyphenols. Appl Microbiol Biotechnol 98:6805-6815

6. Queipo-Ortuño MI, Boto-Ordóñez M, Murri M, Gómez-Zumaquero JM, Clemente-Postigo M, Estruch R, Cardona Díaz F, Andrés-Lacueva C, Tinahones FJ (2012) Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. Am J Clin Nutr 95:1323−1334

7. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD

(2011) Linking long-term dietary patterns with gut microbial enterotypes. Science 334:105–108

8. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R (2009) Bacterial community variation in human body habitats across space and time. Science 326:1694–1697

9. Wade WG (2013) The oral microbiome in health and disease. Pharmacological Research 69:137–143

10. Zarco MF, Vess TJ, Ginsburg GS (2012) The oral microbiome in health and disease and the potential impact on personalized dental medicine. Oral Dis 18:109–120

11. Zaura E, Nicu EA, Krom BP, Keijser BJF (2014) Acquiring and maintaining a normal microbiome: current perspective. Front Cell Infect Microbiol 4:85

12. Cueva C, Moreno-Arribas MV, Martín-Alvarez PJ, Bills G, Vicente MF, Basilio A, Rivas CL, Requena T, Rodríguez JM, Bartolomé B (2010) Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. Res Microbiol 161:372–382

13. Daglia M, Papetti A, Grisoli P, Aceti C, Dacarro C, Gazzani G (2007) Antibacterial activity of red and white wine against oral streptococci. J Agric Food Chem 55:5038−5042

14. Furiga A, Lonvaud-Funel A, Dorignac G, Badet C (2008) *In vitro* anti-bacterial and anti-adherence effects of natural polyphenolic compounds on oral bacteria. J Appl Microbiol 105:1470−1476

15. Muñoz-González I, Thurnheer T, Bartolomé B, Moreno-Arribas MV (2014) Red wine and oenological extracts display antimicrobial effects in an oral bacteria biofilm model. J Agric Food Chem. 62:4731–4737

16. Signoretto, C.; Bianchi, F.; Burlacchini, G.; Sivieri, F.; Spratt, D.; Canepari, P. Drinking Habits Are Associated with Changes in the Dental Plaque Microbial Community. J. Clin. Microbiol. 2010, 48, 347−356.

17. Signoretto C, Canepari P, Stauder M, Vezzuli L, Pruzzo C (2012) Functional foods and strategies contrasting bacterial adhesion. Current Opinion in Biotechnology 23:160–167

18. Varoni EM, Vitalini S, Contino D, Lodi G, Simonetti P, Gardana C, Sardella A, Carrassi A, Iriti M (2013) Effects of red wine intake on human salivary antiradical capacity and total polyphenol content. Food Chem Toxicol 58:289–294

19. Romano-Keeler J, Wynn JL, Maron JL (2014) Great expectorations: the potential of salivary 'omic' approaches in neonatal intensive care. J Perinatol 34:169–173

20. Diaz PI, Dupuy AK, Abusleme L, Reese B, Obergfell C, Choquette L, Dongari-Bagtzoglou A, Peterson DE, Terzi E, Strausbaugh LD (2012) Using high throughput sequencing to explore the biodiversity in oral bacterial communities. Mol Oral Microbiol 27:182–201

21. Hasan NA, Young BA, Minard-Smith AT, Saeed K, Li H, Heizer EM, McMillan NJ, Isom R, Abdullah AS, Bornman DM, Faith SA, Choi SY, Dickens ML, Cebula TA, Colwell RR (2014) Microbial community profiling of human saliva using shotgun metagenomic sequencing. PLoS One 9:e97699

22. Stahringer SS, Clemente JC, Corley RP, Hewitt J, Knights D, Walters WA, Knight R, Krauter KS (2012) Nurture trumps nature in a longitudinal survey of salivary bacterial communities in twins from early adolescence to early adulthood. Genome Res 22: 2146–2152

23. Caufield PW, Li Y, Dasanayake A, Saxena D (2007) Diversity of lactobacilli in the oral cavities of young women with dental caries. Caries Res 41:2–8

24. Washio J, Shimada Y, Yamada M, Sakamaki R, Takahashi N (2014) Effects of pH and lactate on hydrogen sulfide production by oral *Veillonella* spp. Appl Environ Microbiol 80:4184–4188

25. Muñoz-González I, Jiménez-Girón A, Martín-Álvarez PJ, Bartolomé B, Moreno-Arribas MV (2013) Profiling of microbial-derived phenolic metabolites in human feces after moderate red wine intake. J Agric Food Chem 61: 9470–9479

26. Heilig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD, de Vos WM (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol 68: 114–123

27. Satokari RM, Vaughan EE, Akkermans AD, Saarela M, de Vos WM (2001) Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67:504–513

28. Barroso E, Sánchez-Patán F, Martín-Alvarez PJ, Bartolomé B, Moreno-Arribas MV, Peláez C, Requena T, van de Wiele T, Martínez-Cuesta MC (2013) *Lactobacillus plantarum* IFPL935 favors the initial metabolism of red wine polyphenols when added to a colonic microbiota. J Agric Food Chem 61:10163–10172

29. Rasiah IA, Wrong L, Anderson SA, Sissons CH (2005) Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch Oral Biol 50:779–787

30. Cueva C, Mingo S, Muñoz-González I, Bustos I, Requena T, del Campo R, Martin-Alvarez PJ, Bartolomé B, Moreno-Arribas MV (2012) Antibacterial activity of wine phenolic compounds and oenological extracts against potential respiratory pathogens. Lett Appl Microbiol 54:557−563

31. Huys G, Vancanneyt M, D'Haene K, Falsen E, Wauters G, Vandamme P (2007) *Alloscardovia omnicolens* gen. nov., sp. nov., from human clinical samples. Int J Syst Evol Microbiol 57:1442–1446

32. Beighton D, Gilbert SC, Clark D, Mantzourani M, Al-Haboubi M, Ali F, Ransome E, Hodson N, Fenlon M, Zoitopoulos L, Gallagher J (2008) Isolation and identification of Bifidobacteriaceae from human saliva. Appl Environ Microbiol 74:6457–6460

33. Lazarevic V, Whiteson K, Hernandez D, François P, Schrenzel J (2010) Study of inter- and intra-individual variations in the salivary microbiota. BMC Genomics 11:523

34. Clifford MN (2004) Diet-derived phenols in plasma and tissues and their implications for health. Planta Med 70:1103-1114

35. Schwarzberg K, Le R, Bharti B, Lindsay S, Casaburi G, Salvatore F, Saber MH, Alonaizan F, Slots J, Gottlieb RA, Caporaso JG, Kelley ST (2014) The personal human oral microbiome obscures the effects of treatment on periodontal disease. PLoS One 9:e86708

36. Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W, Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J Bacteriol 178:5636−5643

37. Van den Bogert B, de Vos WM, Zoetendal EG, Kleerebezem M (2011) Microarray analysis and barcoded pyrosequencing provide

consistent microbial profiles depending on the source of human intestinal samples. Appl Environ Microbiol 77:2071–2080

38. Rinttilä T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97:1166–1177

39. Lansac N, Picard FJ, Ménard C, Boissinot M, Ouellette M, Roy PH, Bergeron MG (2000) Novel genus-specific PCR-based assays for rapid identification of *Neisseria* species and *Neisseria* meningitidis. Eur J Clin Microbiol Infect Dis 19:443–451

40. Booijink CC, El-Aidy S, Rajilić-Stojanović M, Heilig HG, Troost FJ, Smidt H, Kleerebezem M, De Vos WM, Zoetendal EG (2010) High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol 12:3213–3227

41. Dalwai F, Spratt DA, Pratten J (2007) Use of quantitative PCR and culture methods to characterize ecological flux in bacterial biofilms. J Clin Microbiol 45:3072–3076.

42. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R (2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 68:5445−5451

43. Matar GM, Sidani N, Fayad M, Hadi U. (1998) Two-step PCR-based assay for identification of bacterial etiology of otitis media with effusion in infected Lebanese children. J Clin Microbiol 36:1185–1188

VI.6 SUPPLEMENTARY MATERIAL

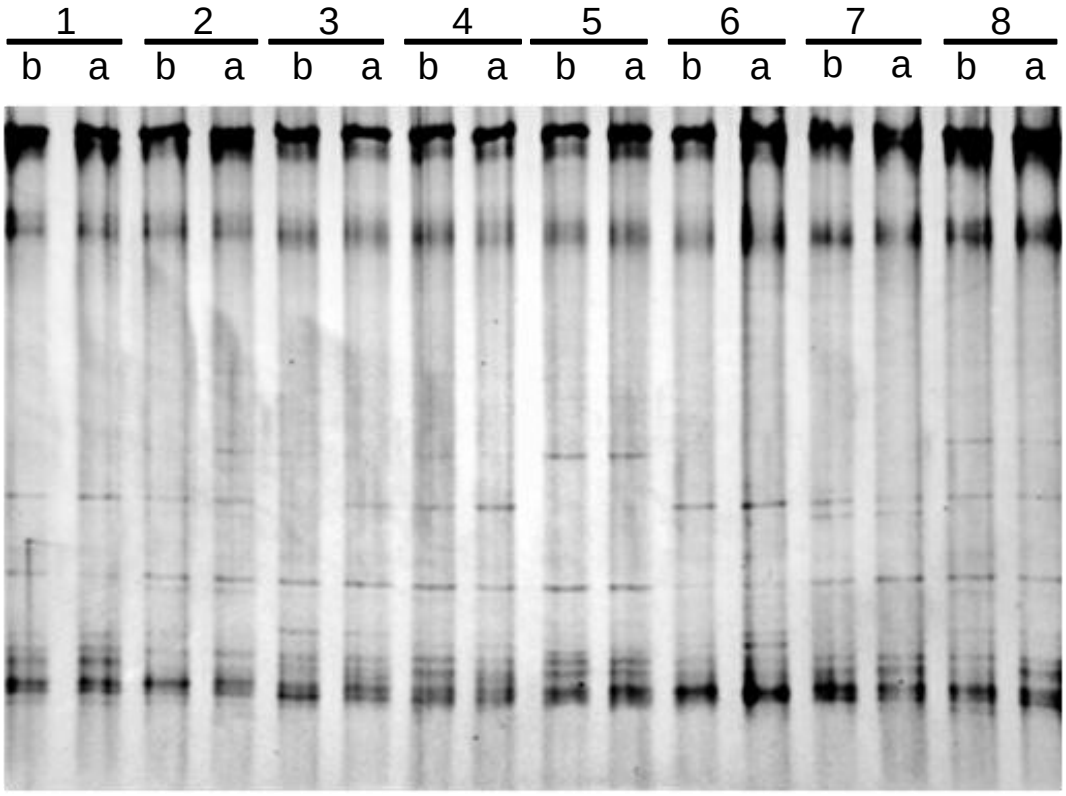

Fig. S1. PCR-DGGE band profiles from eight individuals (out of fourteen) from the intervention group before (b) and after (a) red wine intake obtained from saliva DNA and specific primers for total bacteria.

VII. METAGENOMIC ANALYSIS OF THE INTESTINAL MICROBIOTA IN HEALTHY ADULTS DURING MODERATE INTAKE OF RED WINE

Manuscript in preparation

VII.1 ABSTRACT

There is growing interest in understanding how microbial populations within the human colon can be modified by dietary habits. Here, we examined the influence of moderate red wine intake on the colonic microbiota of 15 healthy volunteers, classified into high, moderate and low polyphenol metabolizers (metabotypes) compared with 5 control subjects. We analyzed the composition, diversity and dynamics of their fecal microbiota before and after one month of wine consumption. The 16S rDNA sequencing allowed detection of 2324 phylotypes, of which only 30 were found over the 0.5% of mean relative frequency, representing 84.6% of the total taxonomical assignments. The samples clustered more strongly by individuals than by wine intake or metabotypes, however an increase in diversity, after the wine intake, was observed when compared with the control volunteers. Some differences in minority microbial groups related with phenolic metabolism were found, but inter-individual variability was the strongest and distinguishing feature. This study confirms the high variability of the microbiota from different individuals, and that the individual microbiota composition seems extremely stable to small dietary changes.

VII.2 INTRODUCTION

The human intestine harbors a microbial community highly diverse that exerts a crucial impact on the human health (Clemente et al., 2012; Tappenden & Deutsch 2007). Of this community, bacteria are the most abundant microorganisms and the colonic part of the intestine the most densely colonized, with approximately 1011–1014 cells/g (Ley et al., 2006; Turnbaugh et al., 2007). The development of culture-independent techniques for microbial community composition analysis has opened new perspectives in understanding the complexity of this human intestinal ecosystem (Yatsunenko et al., 2012; Fraher et al., 2012; Francis & Riley, 2014). Through recent advances in identifying unculturable intestinal communities by using next-generation sequencing technologies, it has been elucidated that the major bacterial phylotypes of the human gut include Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Fusobacteria (Palmer et al., 2007; Lozupone et al., 2012). Due to the implication of the microbiota to the normal host physiology, including proper digestion (del Campo et al., 2014; Salonen & de Vos, 2014), metabolism (Cox et al., 2014) and synthesis of vitamins and co-factors, epithelial cell function (Sommer & Bäckhed, 2013), angiogenesis (Kåhrström, 2012), enteric nerve function (Fernandez et al., 2014) and immune system development (Maslowski et al., 2011), the study of their modulation has become a very important topic.

Recent studies in humans have shown the importance of diet in shaping the gut microbial ecosystem (De Filippo et al., 2010; David et al., 2014) and how intestinal microbiota allows the digestion of food components, otherwise inaccessible to the host enzymes (Salonen & de Vos, 2014). In our diet, different foods and beverages of plant origin, such as red wine, contain secondary metabolites namely polyphenols, claimed as having different biological activities, and consequently an impact on human health. Several epidemiologic studies have related the

consumption of wine or other polyphenols source foods with a lower incidence of cardiovascular disease, hypertension, diabetes, and cancer, partly due to their attributed positive effects on antioxidant capacity, lipid profile and the coagulation system (Diebolt et al., 2001; Lindberg and Amsterdam, 2008; Imhof et al., 2008; Anhê et al., 2014; Seidel el al., 2014; Chen & Sang, 2014). But the *in vivo* effect of polyphenols depends on its bioavailability, absorption and metabolism (Chiou et al., 2014). In this regard, phenolic metabolites produced by bacteria in the human gut seem to be more available than the original forms present in food, thus gut microbiota would enhance their biological activity.

Nevertheless, information about the role of plant polyphenols in the modulation and metabolism of the intestinal ecosystem is scarce. Most of the studies have been performed with single compounds or mixtures of polyphenols using batch-culture fermentation systems inoculated with fecal material (Tzounis et al., 2008; Barroso et al., 2013), and some have used continuous fermentation dynamic models (Kemperman et al., 2013; Barroso et al., 2014). For example, it has been shown that catechin has a positive effect on the growth of the *Blautia coccoides–Eubacterium rectale* group and *Bifidobacterium* while the *Clostridium histolyticum* group was inhibited (Tzounis et al., 2008). In previous *in vitro* studies we have showed that a red wine polyphenol extract affected the growth of *Bacteroides*, *Bifidobacterium* and the *B. coccoides/E. rectale* group (Barroso et al., 2014). This is in agreement with other *in vitro* studies, in which the effect of wine polyphenols also affected the growth of *Bifidobacterium*, *B. coccoides*, *Anaeroglobus*, *Subdoligranulum* and *Bacteroides*, while promoted the growth of *Klebsiella*, *Alistipes*, *Cloacibacillus*, *Victivallis* and *Akkermansia* (Kepermant et al., 2013). Concerning studies *in vivo* of polyphenol containing beverages, a human intervention study (n=8) has shown an increase in qPCR counts of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and *B. coccoides–E. rectale* after the

moderate intake of red wine during 4 weeks (Queipo-Ortuño et al. 2012). But still, more information on the effect of consumption of polyphenol containing beverages, particularly red wine, on the gut microbial ecosystem as a whole and not limited to specific groups of bacteria, is needed.

In this work we have investigated the impact of moderate regular consumption of red wine in the fecal microbial metagenomic profile of healthy individuals. An intervention study was carried out with healthy volunteers consuming red wine for a period of 4 weeks and 16S rRNA gene sequencing of the fecal samples was performed to characterize microbial community changes.

VII.3 MATERIAL AND METHODS

VII.3.1 Wine intervention study

The randomized and controlled study involved a total of 41 healthy volunteers with no recent history of gastrointestinal disease. It was divided in two consecutive periods, an initial washout of 2 weeks, during which the volunteers followed a low-polyphenols diet, and an intervention period of 28 days, in which the volunteers consumed 2 glasses of red wine per day. The control group did not consume wine during the intervention period. More details of the study designs have been published previously (Muñoz-González et al 2013). Fecal samples from all the volunteers were collected before and after the period of intervention and stored at -80ºC. Based on the results of phenolic metabolites obtained by Muñoz-González et al (2013), the volunteers were classified in three metabotypes, regarding the metabolic capability of their microbiota, as low, moderate and high wine-phenolic-metabolizers. In the present study a total of twenty volunteers (five

volunteers from each metabotype and five from the control group) were included.

VII.3.2 Fecal suspension and DNA extraction

Fecal samples were thawed at room temperature, weighted (0.1 g) and suspended in sterile saline solution (NaCl 0.8%) until 1 mL volume. The homogeneous fecal suspension was centrifuged 5 min - 13.000 rpm at 4°C and supernatants were removed. Microbial DNA extraction was performed as described by Moles et al. (2013). The pellet was resuspended in an extraction buffer (200 mM Tris–HCl pH 7.5, 0.5% SDS, 25 mM EDTA, 250 mM NaCl, 20 mg/mL lysozyme, 5 mg/mL lysostaphin and 3 M Na acetate), followed by mechanical lysis with glass beads and extraction with phenol/chloroform/isoamyl-alcohol. The DNA was precipitated by adding 0.6 volumes of isopropanol, washed with 70% ethanol, allowed to air dry, and finally resuspended in DNase, RNase free water (Sigma-Aldrich). The QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) was used to purify and concentrate the DNA. The DNA yield was measured using a NanoDropH ND-1000 UV spectrophotometer (Nano-Drop Technologies) and PicoGreen (Invitrogen).

VII.3.3 16S rRNA gene Illumina MiSeq sequencing

The DNA samples were amplified by PCR using primers 27F-DegL (5'-GTTYGATYMTGGCTCAG-3') in combination with an equimolar mixture of two reverse primers, 338R-I (5'-GCWGCCTCCCGTAGGAGT-3') and 338R-II (5'GCWGCCACCCGTAGGTGT-3') generating an approximately 345 bp amplicons from the V1 to V2 hypervariable regions of 16S rDNA genes. PCR amplification was performed using the Fast Start High Fidelity PCR System dNTP Pack (Roche, Mannheim, Germany) in a total volume of 25 µL containing 2.5 µL 10× Reaction Buffer and 1.25 U of Fast Start High Fidelity Enzyme Blend (Roche), 0.4

µM of each primer, and 10 ng template DNA. Thermal cycling conditions were as follows: an initial denaturation at 95°C for 2 min, and 35 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Following amplification, 2 µL of PCR product was used for agarose gel (1%) detection.

Barcodes used for Illumina sequencing were appended to 3' and 5' terminal ends of PCR amplicons in a second PCR to allow separation of forward and reverse sequences. All primers were synthesized by Isogen Life Sciences (Castelldefels, Spain). Barcoded PCR products from all samples were pooled at approximately equal molar DNA concentrations and run on a preparative agarose gel. The correct sized band was excised and purified using a QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) and quantified with PicoGreen (BMG Labtech, Jena, Germany). The 16S DNA amplicons were sequenced on an Illumina MiSeq (Illumina Inc., San Diego, CA, USA) technology reads 2x250.

VII.3.4 Bioinformatics analysis

In this work each read was assigned to the organism corresponding to the Best Blast Hit (BBH) over a threshold of similarity (e<1E-15) against a global 16S rRNA database specifically built for metagenomic data analysis by Era7 (Granada, Spain). This database contains 902,131 16S rRNA sequences extracted from NCBI nt database because of the specificity of their taxonomical assignment. The direct and cumulative frequencies normalized to the total number of reads assigned to any node of the taxonomy tree have been calculated for every sample.

The richness and diversity of the fecal microbiota were determined by calculating the Shannon-Weaver diversity index, which takes into account the number and evenness of the bacterial species. These calculations were performed using R i386 3.1.0 (R project, Statistical Software).

VII.3.5 Data analysis

The data matrix of the relative frequencies of the microbial groups was used to perform the clustering analysis. To do that, the dendrograms were obtained using the Ward distance agglomeration method after calculating the Kendall coefficients for each sample. The Shapiro-Wilk test was used to assess the normality of the data, using parametric test when variables were normally distributed and the correspondent non-parametric test when not. Principal component analysis (PCA), from the relative frequencies matrix, was performed to study variability among samples and microbial groups and possible trends in the variables due to wine consumption. The comparison of the relative frequencies of the microbial groups, detected at the different taxonomy levels, for the three previously classified metabotypes and the control group before and after wine intake was performed by Anova and the post-hoc LSD test or the correspondent non-parametric Kruskal-Wallis test. To detect microbial significant differences due to wine intake in each group of volunteers, the relative frequencies of each microbial group were analyzed before and after wine intake, with paired t-test when data were normally distributed or the Wilcoxon paired test when not. A value of $P < 0.05$ was fixed for the level of significance of the tests. Statistical analysis was performed in R 2.13.2 (R project, Statistical Software) and STATISTICA program for Windows, version 7.1 (StatSoft Inc., 1984 2006, www.statsoft.com).

VII.4 RESULTS AND DISCUSSION

With the purpose of unravelling the impact of dietary polyphenols in the human intestinal microbiota, we conducted this non-targeted screening of the microbial intestinal content of healthy volunteers after consuming red wine by means of 16S rRNA gene sequencing and phylogenetic analysis of fecal samples. The volunteers were previously grouped in three metabotypes as low, moderate and high wine-phenolic-

metabolizers according to their capacity to metabolize red wine polyphenols (Muñoz-Gonzalez et al., 2013). This work represents the first human red wine intervention study performing a metagenomic analysis to assess the changes in fecal microbiota.

The microbial groups identified in this study were hierarchically clustered according to their phylogenetic level. The relative frequencies of every phylotype for all the samples (before and after the period of study) were analysed. Globally, Firmicutes was the most abundant phylum in all the samples, accounting for approximately 77.3% (Min-max: 47.9%-93.1%) of the total phyla, followed by Actinobacteria (17.3%; 1.9%-49.4%), Bacteroidetes (4.1%; 0.6%-21.2%) and Proteobacteria (1.5%; 0.03%-6.8%) (Fig. VII.1). The class *Clostridia* (Firmicutes) was the most abundant in both control and intervention volunteers accounting for 37.4% (min-max: 22.2-55.1%) of the total classes, followed by *Actinobacteria* class (Actinobacteria) with 17.3% (min-max: 1.9-49.4%) and *Bacilli* (Firmicutes) with 3.4% (min-max: 0.04-19.1%). At genus level, a total of 438 microbial genera were indentified, however, only 15 of them were found in all the samples (Table VII.1). From the total genera, the most abundant were *Bifidobacterium* and *Collinsella* (Actinobacteria) and *Clostridium*, *Ruminococcus* and *Eubacterium* (Firmicutes) (Table VII.1). On a lower taxonomic level, 2324 species-like taxa were detected. Among them, only 30 were found to present a mean relative frequency over the 0.5%, representing an 84.6% of the total taxonomical assignments (Fig VII.2). The dominant taxa in most of the samples corresponded to uncultured Firmicutes sp. except for two volunteers (individuals 8 and 32) in which the dominant taxa were identified as *Catenibacterium* sp. (Firmicutes) and *Streptococcus* sp. (Firmicutes), respectively. *Clostridium* sp., *Peptostreptococcaceae* sp. and *Ruminococcus* sp. showed to be the followed most abundant species in most of the cases. Six *Bifidobacterium* species (*B. longum*, *B. ruminatium*, *B. pseudocatenulatum*, *B. bifidum*, *B. adolescentis* and *B.*

catenulatum) were detected over the 0.5% mean threshold. However, only *B. longum* was present in all the samples. From the total species, only the taxa identified as *Lachnospiraceae*, *Collinsella aerofaciens*, *Clostridiales*, Bacteroidetes, *Eubacterium* sp. and *Streptococcus* sp. were also found in all the samples. To examine the similarity or distance between samples, we performed a cluster analysis using the Kendall correlation coefficient calculated based on the relative frequencies of microbial groups identified over the 0.5% in all samples. The generated dendrogram showed a high individual variability associated to microbial communities, which was stronger than their metabotype or the changes associated to the 4-weeks period of red wine intake (Fig VII.3). This is in agreement with previous evidences of inter-individual variability in the colonic microbiota (Franks et al., 1998; Ley et al., 2006b; Walker et al., 2011).

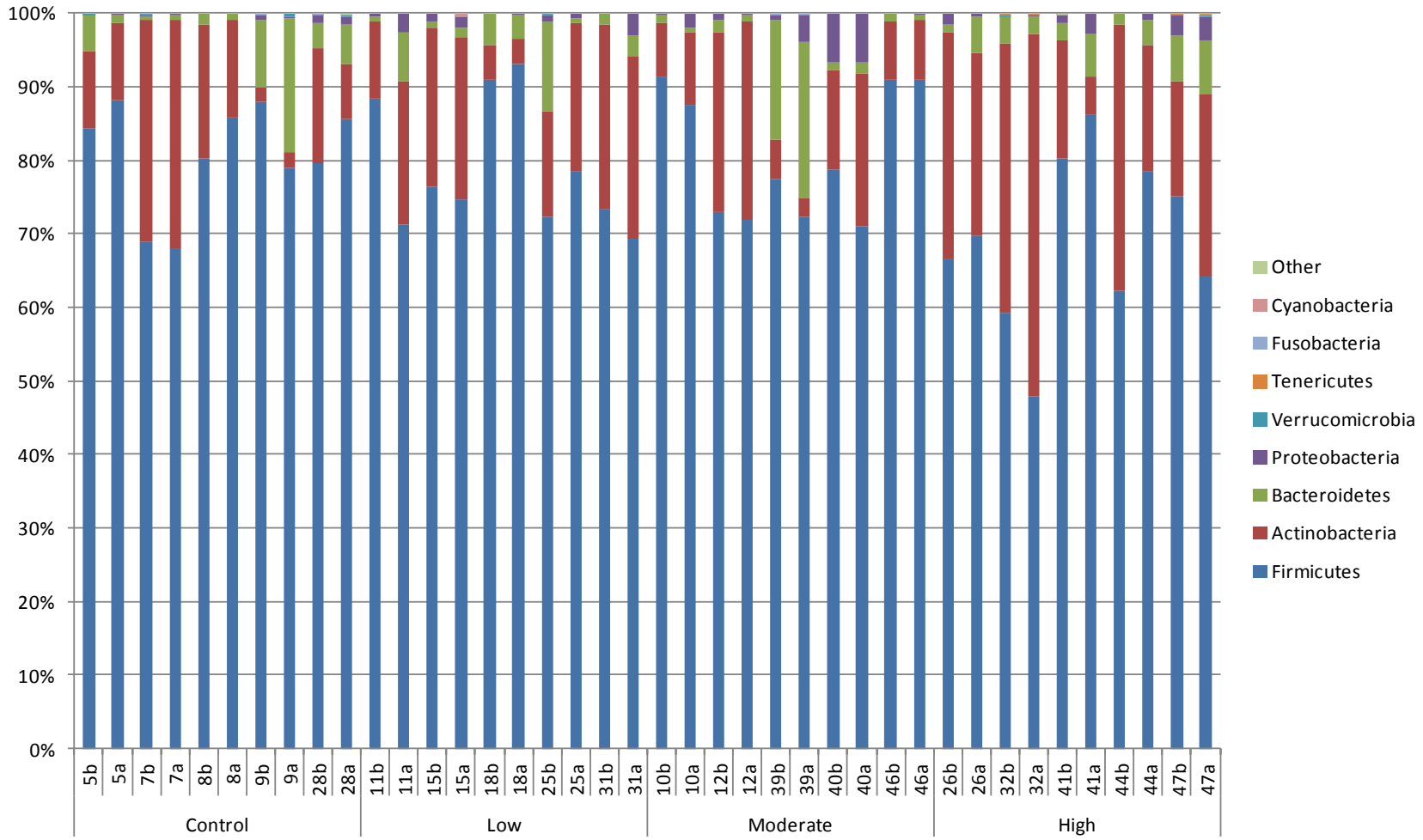

Figure VII.1. Relative frecuencies of the phylum-like phylotypes in the microbiota of feces from volunteers control and low, moderate and high metabotypes. Faecal samples from each volunteer before (b) and after (a) the intervention period are represented with numbers.

Table VII.1. Relative frequencies (%; median) of the genera that were identified in all the samples from volunteers control and low, moderate and high metabotypes before and after the intervention period.

Phylotype, genus	*Phyla*	Before %				After %			
		Control	Low	Moderate	High	Control	Low	Moderate	High
Bifidobacterium	*A*	8.40	10.48	8.02	23.42 *	4.96	14.91	3.11	19.58
Clostridium	*F*	5.43	10.98	4.74	4.94	8.84	5.98	7.51	3.57
Ruminococcus	*F*	7.66	5.26	6.29	4.20	5.71	4.63	5.32	4.80
Eubacterium	*F*	3.62	3.68	2.53	1.08	3.08	2.52	3.72	2.92
Collinsella	*A*	4.64	2.21	2.51	2.64	4.60	3.05	3.23	2.29
Dorea	*F*	0.74	0.77	0.99	0.76	0.77	0.99	1.13	1.33
Roseburia	*F*	0.56	0.88	1.01	0.88	0.68	0.93	0.84	1.11
Sporacetigenium	*F*	1.06	1.12	0.81	0.92	1.08	1.19	0.97	0.79
Streptococcus	*F*	0.62	1.15	0.31	0.74	0.54	3.86	0.85	0.37
Anaerostipes	*F*	0.58	0.78	0.69	0.74	0.76	0.84	1.10	0.32
Faecalibacterium	*F*	0.82	1.06	0.11	1.05	0.73	0.87	0.17	0.46
Bacteroides	*B*	0.14	0.38	0.15	0.21	0.11	0.38	0.22	0.36
Enterococcus	*F*	0.001	0.02	0.08	0.002	0.004	0.02	0.01	0.003
Catenibacterium	*F*	0.003	0.002	0.002	0.001	0.003	0.001	0.003	0.004
Prevotella	*B*	0.001	0.005	0.004	0.003	0.001	0.002	0.01	0.001

F, Firmicutes; B, Bacteroidetes; A, Actinobacteria.
* Differ signifficantly ($P<0.05$) according to ANOVA-test.

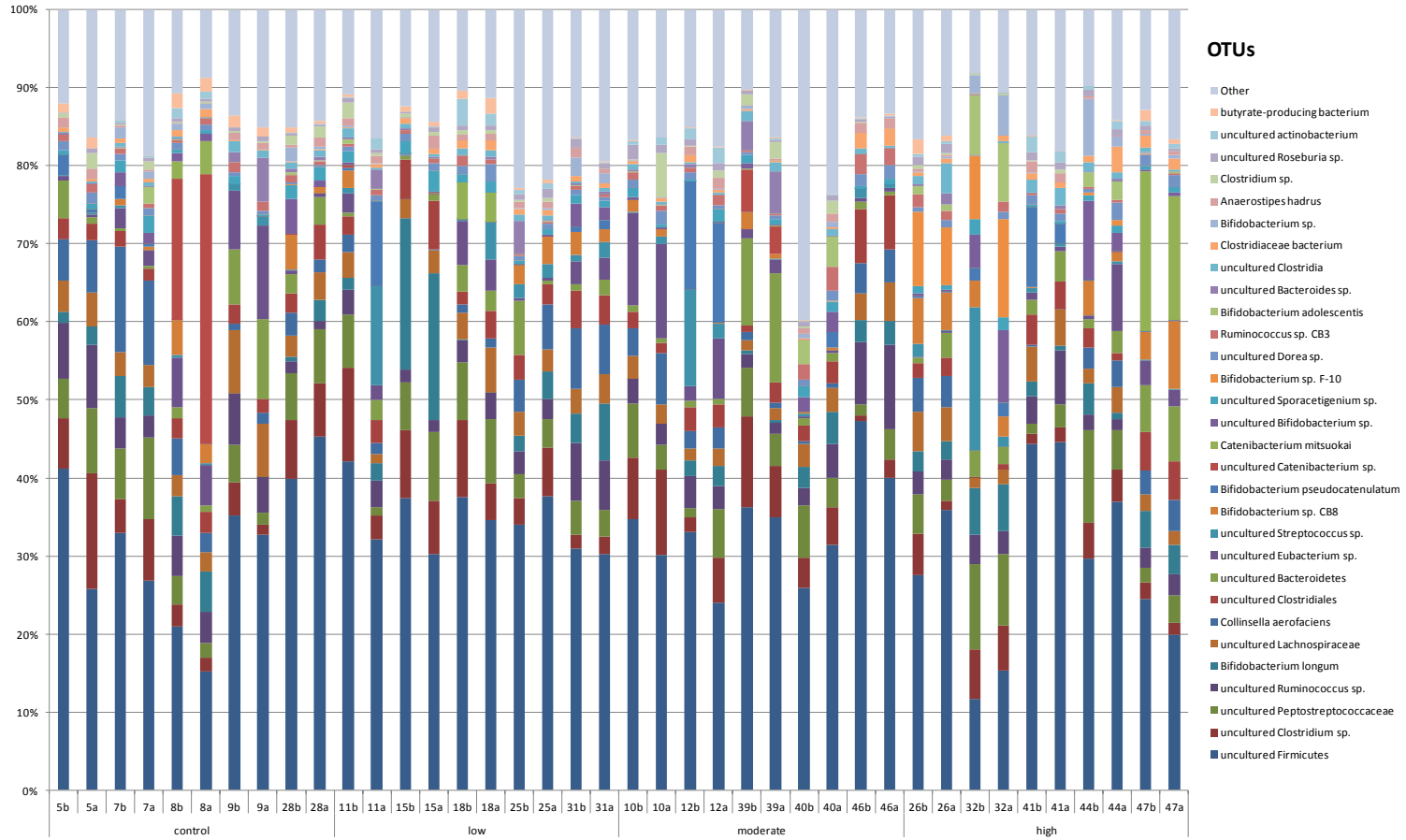

Figure VII.2. Relative frecuencies of the species identified in the microbiota of feces from volunteers control and low, moderate and high metabotypes. Faecal samples from each volunteer before (b) and after (a) the intervention period are represented with numbers.

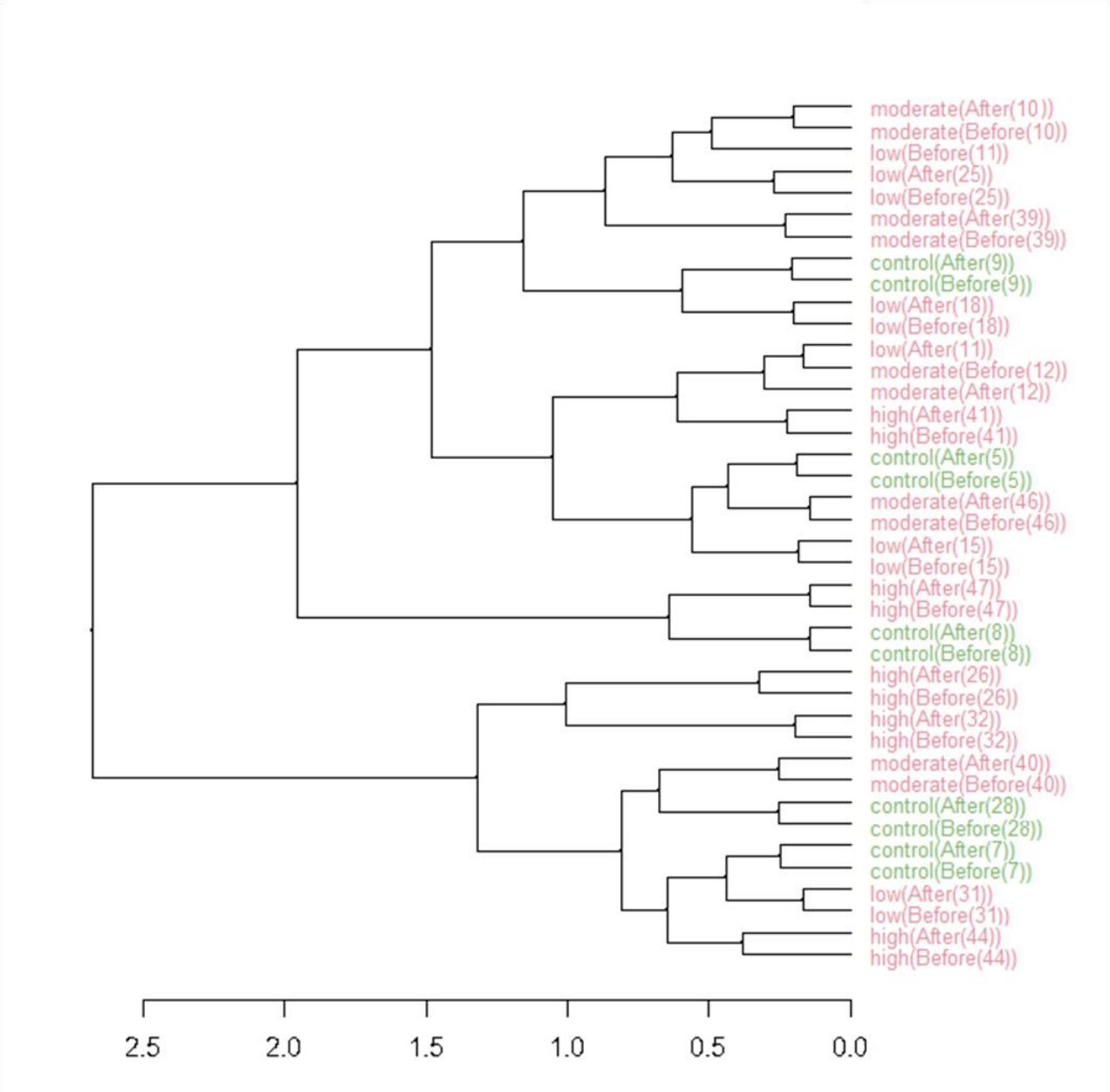

Figure VII.3. Clustering of the bacterial fingerprints of fecal samples from volunteers control (green) and low, moderate and high metabotypes (red) before and after the intervention period. The dendrogram shows the Kendall coefficients for each sample and was obtain using the Ward distance agglomeration method.

The diversity of the microbial communities of the different samples was determined using the Shannon diversity index (SDI). The results obtained showed an average SDI of 2.81±0.14 in the control group (N=5) and 2.81±0.24 in the intervention group (N=15) before the intervention period. The average SDI after the period of study was slightly, but not significantly, lower in the control group (2.79±0.13) than in the intervention group (2.93±0.18). Interestingly, unlike the control group (P=0.851), samples from the intervention group showed significantly higher diversity after the wine intake than before, according to the paired t-test (P=0.007). Conversely when calculating the SDI mean of each metabotype group, the differences before and after the wine intake remain shorter (low, p=0.156; moderate, p=0.037; high, p=0.311), suggesting no correlation of the different metabolic capability with microbial diversity (SDI). In addition, the Anova test of SDI did not group the samples by metabotypes. The overall microbiome differences between intervention and control volunteers and the differences obtained before and after wine consumption were then evaluated by PCA. No separation was observed before and after wine consumption, neither between metabotypes (see in Supporting Information Fig. S1). The principal component 1 (PC1) could explain a 17.5% of the total variance, suggesting that although some differences could be found among the groups of volunteers, the vast majority of the microbiome was not altered due to wine consumption. The PCA also showed the different trend on each volunteer included in this study (Fig VII.4). This reveals the large variability between volunteers. Although volunteers clustered separately, no outliers were noted (Supporting Information Fig. S1). Overall, the observed trends suggest that the responses to the wine intake are influenced by the specific composition of the individual’s intestinal microbiota. Similar results have been observed in other studies, in which changes in non-digestible carbohydrate intake were evaluated (Walker et al., 2011).

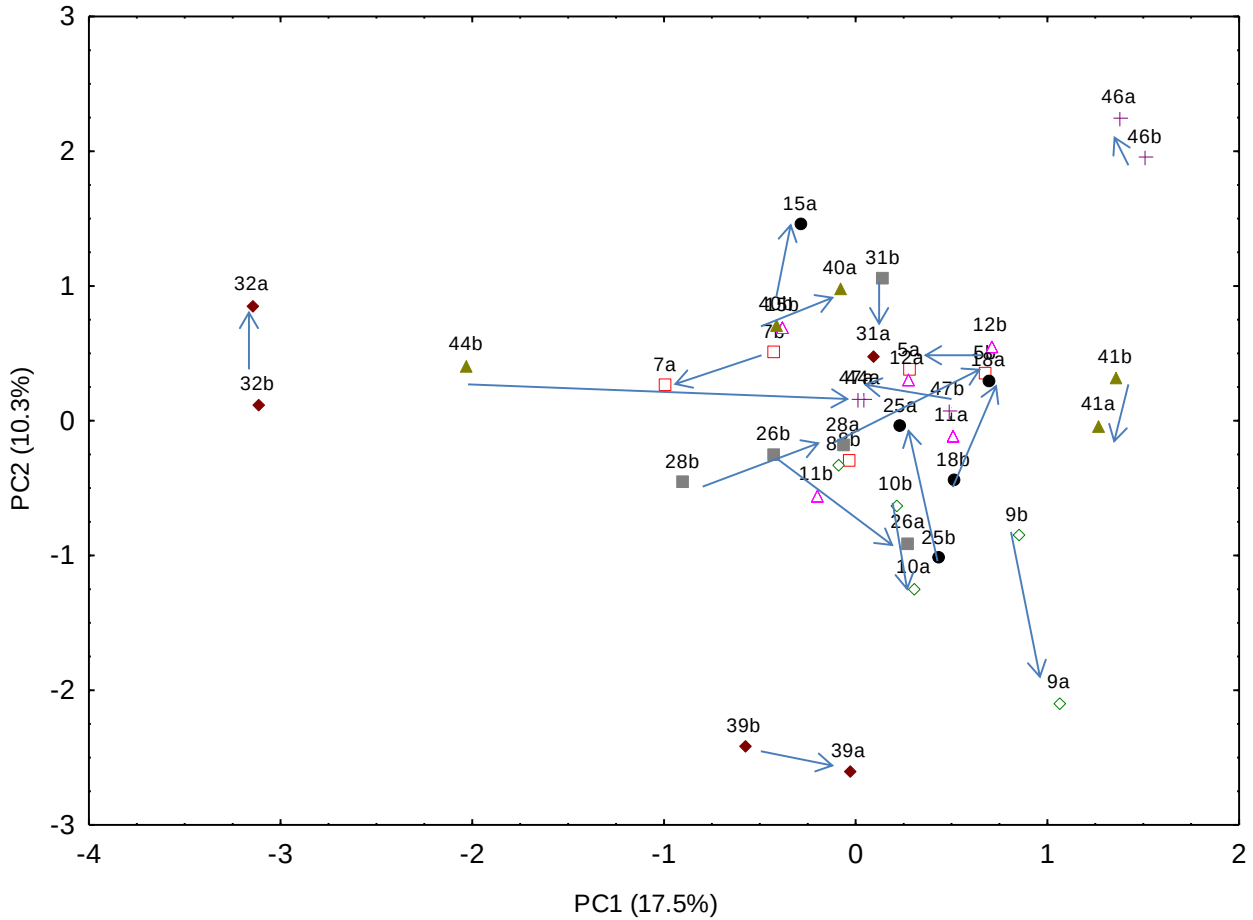

Figure VII.4. Plot of the samples in the plane defined by the two principal components PC1 and PC2 and its response to wine intake indicated by arrows. Faecal samples from each volunteer before (b) and after (a) the intervention period are represented with numbers.

In order to deeper analyze any relation of specific microbial populations with the intake of red wine and the metabolic capability of each group of volunteers, statistic tests of the four most abundant phyla were performed firstly. The results showed that the relative presence in the high metabotype group of Firmicutes (68.68%) and Actinobacteria (27.10%), after the washout period and before the intake of red wine, was significantly different ($P<0.05$) from the other two metabotype groups and the control (Firmicutes: control, 80.3%; low, 80.3% and moderate, 82.3% and Actinobacteria: control, 15.2%; low, 15.3; moderate, 11.6%). In contrast, all of these significant differences disappeared after the period of wine intake. The observed differences in Actinobacteria corresponded at genus level with a higher ($P > 0.05$) relative frequency of *Bifidobacterium* in the high metabotype group than in the others, but only before the intervention period (Table VII.1). These observations could suggest that long term consumption of a moderate amount of red wine could reduce certain differences found between microbiotas from different individuals. However, the disparity in the initial Firmicutes, Actinobacteria and *Bifidobacterium* relative frequencies of the individuals with high metabotype could be due to the small sample of volunteers. This will make necessary more studies with larger number of volunteers in order to confirm some of the responses associated to an extended consumption of wine.

The increased microbial diversity (SDI) observed within the intervention group after the volunteers' wine intake was further analyzed at a lower taxonomic level. The analysis of the 438 genus-like bacterial identified groups showed that some genera present at relative frequencies lower than 0.5% were significantly different ($P>0.05$) after the wine intake, but that these differences were not found in the control group. Among them, there were some genera with documented capability to metabolize polyphenols that increased in the intervention group after the wine intake (Table VII.2). *Slackia* is a genus recently isolated from

feces (Tsuji et al., 2010) that presents trans-resveratrol metabolizing ability (Bode et al., 2013) and contains genes involved in the conversion of isoflavones, daidzein and genistein (Schröder et al., 2013). Other genera that significantly increased after red wine intake were *Oscillatoria*, with previously reported metabolic capabilities such laccase, polyphenol oxidase, peroxidase and esterase (Viswajith and Malliga, 2008; Prabha et al., 2010), *Gordonibacter*, which has showed capabilities for ellagic acid catabolism and urolithin formation (Selma et al., 2014), and *Veillonella* that has been related with the intestinal metabolism of dietary daidzein into equol (Decroos et al., 2005). The genus *Oenococcus* was also detected in higher proportions after the wine intake and due to its role as responsible for malolactic fermentation in wines (Dicks et al., 1995), its increase could be related to its presence in the red wine ingested by the volunteers.

The findings reported in this study can provide approaches about the modulation mechanisms of the wine intake into microbial diversity, suggesting an increase in diversity in terms of relative frequencies of microbial groups. This assumption probably requires future studies with higher number of volunteers in order to confirm that the shifts observed are mainly due to the inclusion of red wine intake in the diet. The association of dietary changes to the colonic microbial population's structure is an emerging concept (Korpela et al., 2014), however, the individual differences in the response associated with diet are acknowledged as intrinsic trait of the human intestinal microbiota (Martínez et al., 2010; Walker et al., 2011; Lampe et al., 2013). In addition, the individual microbiota composition seems extremely stable to small dietary changes.

Table VII.2. Relative frecuencies (%; median with minimum and maximum values in brackets) of the genera identified in the microbiota of feces from volunteers that showed significant differences after the wine intake.

	Control group				Wine group			
	N	Initial	Final	*P*	N	Initial	Final	*P*
Slackia	5	0.0120 (0.0009-0.2650)	0.0119 (0.0029-0.1811)	0.138	13	0.0045 (0.0000-0.0725)	0.0127 (0.0007-0.1189)	0.001
Gordonibacter	4	0.0027 (0.0010-0.0133)	0.0061 (0.0020-0.0517)	0.465	13	0.0006 (0.0000-0.0346)	0.0021 (0.0000-0.0674)	0.046
Oscillatoria	3	0.0005 (0.0000-0.0033)	0.0017 (0.0015-0.0302)	0.593	11	0.0000 (0.0000-0.0028)	0.0016 (0.0000-0.3750)	0.026
Veillonella	2	0.0014 (0.0010-0.0019)	0.0038 (0.0020-0.0056)	-	13	0.0009 (0.0000-0.0069)	0.0010 (0.0000-0.0116)	0.023
Oenococcus	1	0.0000 (0.0000-0.0000)	0.0015 (0.0015-0.0015)	-	6	0.0000 (0.0000-0.0006)	0.0006 (0.0000-0.0036)	0.074

P values evaluated with the Wilcoxon paired-test.

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VII.5 REFERENCES

Agans R., Rigsbee L., Kenche H., Michail S., Khamis H.J., Paliy O. (2011). Distal gut microbiota of adolescent children is different from that of adults. FEMS Microbiol. Ecology, 77, 404-412.

Anhê F.F., Roy D., Pilon G., Dudonné S., Matamoros S., Varin T.V., Garofalo C., Moine Q., Desjardins Y., Levy E., Marette A. (2014). A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased *Akkermansia* spp. population in the gut microbiota of mice. Gut, 307142.

Arranz S., Chiva-Blanch G., Valderas-Martínez P., Medina-Remón A., Lamuela Raventós R.M., Estruch R. (2012). Wine, beer, alcohol and polyphenols on cardiovascular disease and dancer. Nutrients, 4, 759-781.

Artis D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut . Nat. Rev. Immunol., 8, 411–420.

Backhed F., Ding H., Wang T., Hooper L.V., Koh G.Y., Nagy A., Semenkovich C.F., Gordon J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage . Proc. Natl. Acad. Sci. USA, 101, 15718-15723.

Barroso E., Van de Wiele T., Jiménez-Girón A., Muñoz-González I., Martín-Alvarez P.J., Moreno-Arribas M.V., Bartolomé B., Peláez C., Martínez-Cuesta M.C., Requena, T. (2014). *Lactobacillus plantarum* IFPL935 impacts colonic metabolism in a simulator of the human gut microbiota during feeding with red wine polyphenols. Appl. Microbiol. Biotechnol, 98, 6805-6815.

Berg G., Eberl L., Hartmann A. (2005). The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Environ Microbiol., 7, 1673-1685.

Bode L.M., Bunzel D., Huch M., Cho G.S., Ruhland D., Bunzel M., Bub A., Franz C.M., Kulling S.E. (2013). *In vivo* and *in vitro* metabolism of trans-resveratrol by human gut microbiota. Am. J. Clin. Nutr., 97, 295-309.

Bolca S., van de Wiele T., Possemiers S. (2013). Gut metabotypes govern health effects of dietary polyphenols. Curr. Opin. Biotechnol. 24, 220−225.

Chen H., Sang S. (2014). Biotransformation of tea polyphenols by gut microbiota. J. Func. Foods, 7, 26-42.

Chiou Y.S., Wu J.C., Huang Q., Shahidi F., Wang Y.J., Ho, C.T., Pan M.H. (2014). Metabolic and colonic microbiota transformation may enhance the bioactivities of dietary polyphenols. J. Func. Foods, 7, 3-25.

Clemente J.C., Ursell L.K., Parfrey L.W., Knight R. (2012). The impact of the gut microbiota on human health: an integrative view. Cell, 148, 1258-1270.

Contreras A., Doan N., Chen C., Rusitanonta T., Flynn M.J., Slots J. (2000). Importance of *Dialister pneumosintes* in human periodontitis. Oral Microbiol Immunol., 15, 269-272.

Cox L.M., Yamanishi S., Sohn J., Alekseyenko A.V., Leung J.M., Cho I., G. Kim S.G., Li H., Gao Z., Mahana D., Rodriguez J.G.Z., Rogers A.B., Robine N., Loke P., Blaser, M. J. (2014). Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell, 158, 705-721.

Cueva C., Bartolomé B., Moreno-Arribas M. V., Bustos I., Requena T., González-Manzano S., Santos-Buelga C., Turrientes M.C., del Campo R. (2014). Susceptibility and tolerance of human gut culturable aerobic microbiota to wine polyphenols. Microb. Drug Resist., 21, 17-24.

David L.A., Maurice C.F., Carmody R.N., Gootenberg D.B., Button J.E., Wolfe B.E, Ling A.V, Devlin A.S., Varma Y., Fischbach M.A., Biddinger S.B., Dutton R.J., Turnbaugh P.J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature, 505, 559–563.

De Filippo C., Cavalieri D., Di Paola M., Ramazzotti M., Poullet J. P., Massart S., Collini S., Pieraccini G., Lionetti P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc. Natl. Acad. Sci. U.S.A., 107, 14691-14696.

Decroos K., Vanhemmens S., Cattoir S., Boon N., Verstraete W. (2005). Isolation and characterisation of an equol-producing mixed microbial culture from a human faecal sample and its activity under gastrointestinal conditions. Arch. Microbiol., 183, 45-55.

Del Campo R., Garriga M., Pérez-Aragón A., Guallarte P., Lamas A., Máiz L., Bayón C., Roy G., Cantón R., Zamora J., Baquero F., Suárez L. (2014). Improvement of digestive health and reduction in proteobacterial populations in the gut microbiota of cystic fibrosis patients using a *Lactobacillus reuteri* probiotic preparation: A double blind prospective study. J. Cyst. Fibros., 13, 716-722.

Dethlefsen L., Huse S., Sogin M.L., Relman D.A. (2008). The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol., 6:e280.

Dicks L.M.T., Dellaglio F., Collins M.D. (1995). Proposal to reclassify Leuconostoc oenos as *Oenococcus oeni* [corrig.] gen. nov., comb. nov. Int.J. Syst. Bacteriolo., 45, 395-397.

Diebolt M., Bucher B., Andriantsitohaina R. (2001). Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. Hypertension, 38, 159–165.

Fernandez L.M.B., Lasa J.S. Man F. (2014). Intestinal microbiota: Its role in digestive diseases. J. Clin. Gastroenterol., 48, 657-666.

Foligné B., Dewulf J., Breton J., Claisse O., Lonvaud-Funel A., Pot B. (2010). Probiotic properties of non-conventional lactic acid bacteria: Immunomodulation by *Oenococcus oeni*. Int. J. Food Microbiol., 140, 136-145.

Fraher M.H., O'Toole P.W., Quigley E.M. (2012). Techniques used to characterize the gut microbiota: a guide for the clinician. Nat. Rev. Gastroentero, 9, 312-322.

Francis S.S., Riley L.W. (2014). Metagenomic epidemiology: a new frontier. J. Epidemiol. Community Health, 203997.

Franke A.A., Custer L.J., Cooney R.V., Tanaka Y., Xu M., Dashwood R.H. (2002). Inhibition of colonic aberrant crypt formation by the dietary flavonoids (+)–catechin and hesperidin. Adv. Exp. Med. Biol., 505, 123–133.

Franks A.H., Harmsen H.J.M., Raangs G.C., Jansen G.J., Schut F., Welling G.W. (1998). Variations of bacterial populations in human feces measured by fluorescent in situ hybridisation with group-specific 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol., 64, 3336–3345.

Hooper L.V., Midtvedt T., Gordon J.I. (2002). How host - microbial interactions shape the nutrient environment of the mammalian intestine . Annu. Rev. Nutr., 22, 283-307.

Imhof A., Blagieva R., Marx N., Wolfgang K. (2008). Drinking modulates monocyte migration in healthy subjects: a randomised intervention study of water, ethanol, red wine and beer with or without alcohol. Diab. Vasc. Dis. Res., 5, 48–53.

Kåhrström C.T. (2012). Host response: Pro-angiogenic bacteria in the gut. Nat. Rev. Microbiol., 10, 310-310.

Kemperman R.A., Gross G., Mondot S., Possemiers S., Marzorati M., Van de Wiele T., Doré J., Vaughan E.E. (2013). Impact of polyphenols from black tea and red wine/grape juice on a gut model microbiome. Food Res. Int., 53, 659-669.

Korpela K., Flint H., Johnstone A.M., Lappi J., Poutanen K., Dewulf E. Delzenne N., de Vos W.M, Salonen A. (2014). Gut microbiota signatures predict host and microbiota responses to dietary interventions in obese individuals. PLoS One, 9, e90702.

Lampe J.W., Navarro S.L., Hullar M.A.J., Shojaie A. (2013). Inter-individual differences in response to dietary intervention: integrating omics platforms towards personalized dietary recommendation. Proc. Nutr. Soc., 72, 207–218.

Lee H.C., Jenner A.M., Low C.S., Lee Y.K. (2006). Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. Res. Microbiol., 157, 876–884.

Ley R.E., Peterson D.A., Gordon J.I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell, 124, 837-848.

Ley R.E., Turnbaugh P.J., Klein S., Gordon J.I. (2006b). Microbial ecology-human gut microbes associated with obesity. Nature, 444, 1022–1023.

Lindberg M.L., Amsterdam E.A. (2008). Alcohol, wine, and cardiovascular health. Clin. Cardiol., 31, 347–351.

Lozupone C.A., Stombaugh J.I., Gordon J.I., Jansson J.K., Knight R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature, 489, 220-230.

Marques T.M., Cryan J.F., Shanahan F., Fitzgerald G.F., Ross R.P., Dinan T.G., Stanton C. (2014). Gut microbiota modulation and implications for host health: Dietary strategies to influence the gut–brain axis. Innov. Food Sci. Emerg., 22, 239-247.

Martínez I., Kim J., Duffy P.R., Schlegel V.L., Walter J. (2010). Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. PLoS One, 5, e15046.

Maslowski K.M., Mackay C.R. (2011). Diet, gut microbiota and immune responses. Nat. immunol., 12, 5-9.

Morotomi M., Nagai F., Sakon H., Tanaka R. (2008). Dialister succinatiphilus sp. nov. and Barnesiella intestinihominis sp. nov., isolated from human faeces. Int. J. Syst. Evol. Microbiol., 58, 2716-2720.

Muñoz-González I., Jiménez-Girón A., Martín-Álvarez P.J., Bartolomé B., Moreno-Arribas M.V. (2013). Profiling of microbial-derived phenolic metabolites in human feces after moderate red wine intake. J. Agric. Food Chem., 61, 9470-9479.

O'Toole P.W., Claesson M.J. (2010). Gut microbiota: changes throughout the lifespan from infancy to elderly. Int. Dairy J., 20, 281-291.

Palmer C., Bik E.M., DiGiulio D.B., Relman D.A., Brown P.O. (2007). Development of the human infant intestinal microbiota. PLoS Biol., 5:e177.

Piot P., Van Dyck E., Totten P.A., Holmes K.K. (1982). Identification of *Gardnerella* (*Haemophilus*) *vaginalis*. J. Clin. Microbiol., 15, 19-24.

Pozo-Bayón M. A., Monagas M., Bartolomé B., Moreno-Arribas M. V. (2012). Wine features related to safety and consumer health: an integrated perspective. Crit. Rev. Food Sci. Nutr., 52, 31-54.

Prabha D.S., Karthikeyan K., Navanietha K.R., Akila B.M., Hemanth S., Karikrishnan R., Archunan G., Malliga, P. (2010). Effect of phenolic compounds released during degradation of coir pith by *Oscillatoria annae* on albino rat (Rattus norvegicus). J. Appl. Sci. Environ. Manag., 13(4).

Queipo-Ortuño M.I., Boto-Ordoñez M., Murri M., Gomez-Zumaquero J.M., Clemente-Postigo M., Estruch R., Cardona Diaz F., Andrés-Lacueva C., Tinahones F.J. (2012). Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. Am. J. Clin. Nutr., 95, 1323-1334.

Rajilić-Stojanović M., Smidt H., de Vos W.M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. Environ. Microbiol., 9:2125–2136.

Requena T., Monagas M., Pozo-Bayon M.A., Martin-Alvarez P.J., Bartolome B., del Campo R., Avila M., Martinez-Cuesta M.C., Pelaez C., Moreno-Arribas M.V. (2012). Perspectives of the potential implications of wine polyphenols on human oral and gut microbiota. Trends Food Sci. Technol., 21, 332-344.

Salonen A., de Vos W.M. (2014). Impact of diet on human intestinal microbiota and health. Annu. Rev. Food Sci. Technol., 5, 239-262.

Schröder C., Matthies A., Engst W., Blaut M., Braune A. (2013). Identification and expression of genes involved in the conversion of daidzein and genistein by the equol-forming bacterium *Slackia* isoflavoniconvertens. Appl. Environ. Microbiol., 79 (11), 3494-3502.

Seidel D., Martnez I., Taddeo S., Joseph M., Carroll R., Haub M., Walter J., Turner, N. (2014). A polyphenol-rich sorghum cereal alters colon microbiota and plasma metabolites in overweight subjects. The FASEB Journal, 28 (1 Supplement), 270-7.

Selma M.V., Espin J.C., Tomas-Barberan F.A. (2009) Interaction between phenolics and gut microbiota: role in human health. J. Agric. Food Chem., 57, 6485-6501.

Selma M.V., Garcia-Villalba R., Espín J.C., Tomás-Barberán F.A. (2014). Description of urolithin production capacity from ellagic acid of two human intestinal bacteria species of *Gordonibacter* genus. Food Funct., 5, 1779-1784.

Siddharth J., Holway N., Parkinson S.J. (2013). A western diet ecological module identified from the 'humanized'mouse microbiota predicts diet in adults and formula feeding in children. PloS One, 8, e83689.

Sommer F., Bäckhed F. (2013). The gut microbiota-masters of host development and physiology. Nat. Rev. Microbiol., 11, 227-238.

Spellerberg I.F., Fedor P.J. (2003). A tribute to Claude-Shannon (1916-2001) and a plea for more rigorous use of species richness, species diversity and the "Shannon-Wiener" Index. Global Ecology & Biogeography, 12, 177–179.

Swidsinski A., L*oeni*ng-Baucke V., Mendling W., Dörffel Y., Schilling J., Halwani Z., Jiang X.F., Verstraelen H., Swidsinski S. (2014). Infection through structured polymicrobial *Gardnerella* biofilms (StPM-GB). Histol. Histopathol., 29, 567-587.

Tap J., Mondot S., Levenez F., Pelletier E., Caron C., Furet J. P., Ugarte E., Muñoz Tamayo R., Paslier D.L.E., Nalin R., Dore J., Leclerc, M. (2009). Towards the human intestinal microbiota phylogenetic core. Environ. Microbiol., 11, 2574-2584.

Tappenden K.A., Deutsch A.S. (2007). The physiological relevance of the intestinal microbiota, contributions to human health. J Am Coll Nutr. 26, 679S–683S.

Tsuji H., Moriyama K., Nomoto K., Miyanaga N., Akaza H. (2010). Isolation and characterization of the equol-producing bacterium *Slackia* sp. strain NATTS. Arch. Microb., 192, 279-287.

Turnbaugh P.J., Ley R.E., Hamady M., Fraser-Liggett C., Knight R., Gordon J.I. (2007). The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature, 449, 804-810.

Tzounis X., Rodriguez-Mateos A., Vulevic J., Gibson G.R., Kwik-Uribe C., Spencer J.P.E. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. Am. J. Clin. Nutr., 93, 62-72.

Tzounis X., Vulevic J., Kuhnle G.G., George T., Leonczak J., Gibson G.R., Kwik-Uribe C., Spencer J.P. (2008). Flavanol monomer-induced changes to the human faecal microflora. Br. J. Nutr., 99, 782–792.

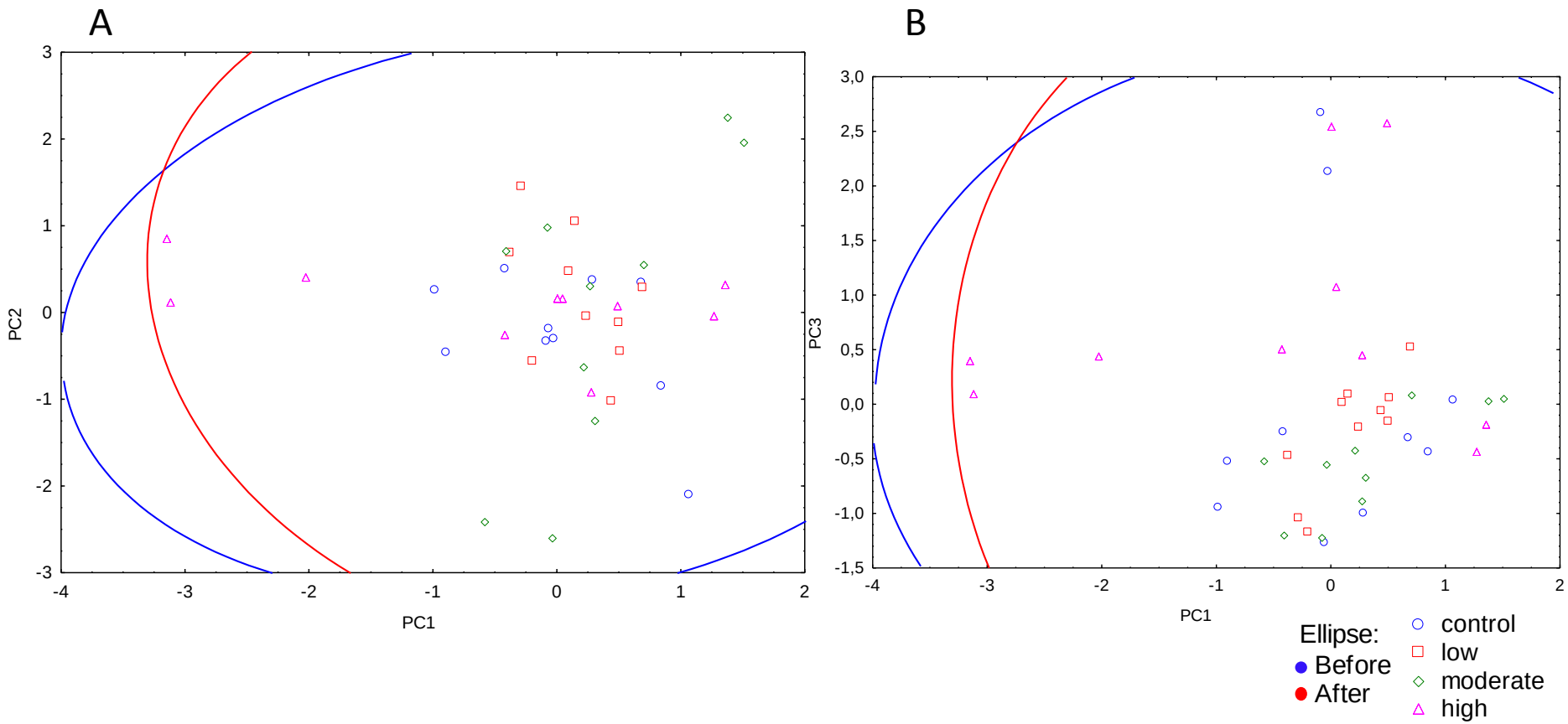
Viswajith V., Malliga P. (2008). Lignolytic enzyme profile of *Oscillatoria annae* in response to Lantana camara. Indian J BotRes., 4, 275-278.

Walker A.W., Ince J., Duncan S.H., Webster L.M., Holtrop G., Ze X., Brown D., Stares M.D., Scott P., Bergerat A., Louis P., McIntosh F., Johnstone A.M., Lobley G.E., Parkhill J., Flint H.J. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J., 5, 220-230.

Yatsunenko T., Rey F.E., Manary M.J., Trehan I., Dominguez-Bello M.G., Contreras M., Magda Magris M., Hidalgo G., Baldassano R.N., Anokhin A.P., Heath A.C., Warner B., Reeder J., Kuczynski J., Caporaso J.G., Lozupone C.A., Lauber C., Clemente J.C., Knights D., Knight R., Gordon, J. I. (2012). Human gut microbiome viewed across age and geography. Nature, 486, 222-227.

Zoetendal E.G, Rajilc´-Stojanovic´ M, de Vos W.M. (2008). High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. Gut, 57, 1605–1615.

Zoetendal E.G., Akkermans A.D., de Vos W.M. (1998). Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol., 64, 3854–3859.



Supporting information Fig. S 1. Plot of the samples in the plane defined by (A) the two principal components (PC1 and PC2) and defined by (B) the PC1 and PC3. Faecal samples from volunteers control, low, moderate and high metabotypes are represented in blue circles, red squares, green diamonds and pink triangles, respectively. 99% colored confidence ellipses representing before (blue) and after (red) wine consumption are also included. The percentages of the total variance explained by each component, PC1, PC2 and PC3 are 17.54, 10.34 and 9.83 respectively.

VIII. DEVELOPMENT OF HUMAN COLONIC MICROBIOTA IN THE COMPUTER-CONTROLLED DYNAMIC SIMULATOR OF THE GASTROINTESTINAL TRACT (SIMGI)

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Barroso E., Cueva C., Peláez C., Martínez-Cuesta M. C., Requena T. (2015). Development of human colonic microbiota in the computer-controlled dynamic SIMulator of the GastroIntestinal tract SIMGI. LWT Journal of Food Science and Technology, 61, 283-289.

VIII.1 ABSTRACT

This study presents a unique fully computer-controlled dynamic SIMulator of the GastroIntestinal tract, SIMGI, which has been designed to simulate the complete processes of digestion and fermentation. This model is programmable to sequentially proceed (continuously or feeding the system from 1 to 6 times daily) from the operation of food intake into the stomach throughout the delivery of distal colon content to waste. The system includes three-stage culture reactors that are capable to reproduce *in vitro* the microbial conditions that characterize the different regions of the human large intestine. The evolution and composition of the microbial community in the ascending (AC), transverse (TC) and descending colon (DC) vessels was evaluated by PCR-DGGE and real-time PCR. An overall decrease in counts of *Bifidobacterium* and *Prevotella* and an increase of *Enterobacteriaceae* was observed between the inoculation with human faeces and the bacterial community stabilized in the colon vessels after two weeks. Regarding microbial differentiation, *Bacteroides* counts were more representative of the AC and TC vessels than the DC compartment. Within the butyrate producer groups, a lower occurrence of *Clostridium leptum* and Ruminoccocus was observed in the AC compartment than in the TC and DC vessels. Accordingly, the net SCFA production was highest in the AC compartment, whereas the results of ammonium formation indicated that proteolysis occurred similarly throughout the entire colon compartments. The results of microbial stabilization observed in the new dynamic *in vitro* model SIMGI indicate that the system can be used as a tool for studying the effects of diet or food components on modulating the gut microbiota and its metabolic activity.

VIII.2 INTRODUCTION

Food digestion and fermentation are complex processes that take place through the gastrointestinal tract (GIT). Food digestion starts in the mouth and continues in the stomach and the small intestine where most of the available food components are absorbed. Many of the indigestible components of the diet, like complex carbohydrates or polyphenols, reach intact the large intestine where can partially be absorbed after being metabolized by the resident colonic microbiota (Possemiers et al. 2011). The human intestinal microbiota is a highly complex and dynamic ecosystem that harbors around 500-1000 microbial species (typically 1011-1012 microbes/g of luminal colon content). Besides its metabolic role on the indigestible fractions of the diet, the gut microbiota serves for numerous important functions for human health, including the maintenance of intestinal homeostasis (Hooper et al. 2012). Several factors such as diet, genetic background, and immune status affect the composition and metabolism of the gut microbiota (Benson et al. 2010; Wu et al. 2011). Among them, diet appears to be one of the most important factors influencing the mid-long term microbial metabolism (David et al. 2014), and therefore can be crucial in understanding many health benefits related to this complex community.

The *in vivo* study of the human GIT functions and its environment, in both health and disease conditions, is limited by ethical concerns and is not acceptable when potentially harmful substances are involved. Therefore, several *in vitro* models have been developed to closely simulate the complex multistage processes of human digestion and to dynamically monitor the microbial processes at the site of metabolic activity (Fritz et al. 2013; Guerra et al. 2012; Venema and Van den Abbeele 2013). These *in vitro* systems have allowed the screening of a large number of conditions, studying the separate and combined impacts of each stage of digestion and fermentation. Dynamic gastric models

have been developed and designed for real time measurement of the effects of the biochemical and mechanical processing of foods in the stomach (Kong and Singh 2010; Wickham and Faulks 2012). The TNO gastrointestinal model (TIM-1) is a computerized dynamic system that combines the physiological processes occurring within the stomach and the three parts of the small intestine (Minekus et al. 1995). As well as for the digestion models, the complexity of the *in vitro* fermentative models is diverse, ranging from batch fecal incubations using anaerobic conditions and dense fecal microbiota suitable for metabolic studies (Aura et al. 2012) to more complex multi-compartmental models that represent different parts of the human colon and allow characterization of different gut microbial species and their related functionality over long-term periods. Multi-compartmental models are usually represented by three-stage culture reactors as that designed by Gibson et al. (1988) that can reproduce differences from proximal (low pH, carbohydrate-excess conditions) to distal colonic regions (carbohydrate-depleted, non-acidic environment). Furthermore, the SHIME (Simulator of the Human Intestinal Microbial Ecosystem) adds two additional reactors representing the gastric and duodenal stages that follow a fill and flow process (Molly et al. 1993). The duodenal content is pumped to three connected colonic reactors, which keep constant volumes in order to define residence times and have control of pH to allow differentiation of colon region-specific microbiota (Van den Abbeele et al. 2010). A three-stage model using immobilized microbiota in xanthan-gellan gum gel beads has been developed to represent the planktonic and sessile states of the complex colonic bacterial community (Cinquin et al. 2006). The TIM-2 is a dynamic computer-controlled model of the proximal colon (Minekus et al. 1999) that uses peristaltic movements to mix and transport the colonic content. It includes a dialysis process that simulates passive absorption of microbial metabolites and water, allowing the system to encompass a high-density microbiota. Although the TIM-1 and TIM-2 are automated

complementary models representing the upper and distal GIT, respectively, they are usually not connected to operate jointly (Hatanaka et al. 2012).

Therefore, most of the models have been specialized either in simulating the upper gastric-small intestine digestion or the colonic fermentation process involving gut microbiota. The dynamic SIMulator of the GastroIntestinal tract SIMGI described in this article goes one step further and represents a fully computer-controlled multi-compartmental system, which allows joint or separated simulation of the gastric and colonic fermentative processes. Thus, the SIMGI is a flexible modulating system that combines a gastric compartment that simulates peristaltic mixing movements, a reactor simulating the small intestine and three-stage continuous reactors for reproducing the colon region-specific microbiota and its metabolism. Besides the engineered functioning of the five SIMGI compartments, the biological functioning of the system requires the development of a colon region-specific microbial ecosystem that needs to be stabilized in the simulator before starting any experimental approach. The microbiota stabilization observed in this model allows the system to be used as a tool for studying the effects of diet or food components on the modulation of the GIT microbiota and its metabolic activity.

VIII.3 MATERIALS AND METHODS

VIII.3.1 Description of the SIMGI Model

The SIMGI comprises of five compartments (units), simulating the stomach, small intestine (SI) and the ascending (AC), transverse (TC) and descending colon (DC) regions, which are interconnected by pipes and peristaltic valve pumps (Watson-Marlow 120 U/DV) that transfer the content between the successive units (Fig. VIII.1). The whole system is

computer controlled through an operator panel and programmable logic controller (Unitronics Vision120™). The stomach is comprised of two transparent and rigid methacrylate plastic modules covering a reservoir of flexible silicone walls where the gastric content is mixed by peristaltic movements. The gastric peristalsis is achieved by pumping thermostated water that flows in the jacket between the plastic modules and the flexible reservoir, and that additionally keeps the temperature of the gastric content at 37 °C. The meal received by the stomach compartment is mixed with gastric electrolytes and enzymes and the decrease of pH is controlled by adding 0.5 M HCl. The computer software (SIMGI, Bioprocess Technology, Spain) allows the definition of pH acidification curves and the control of emptying times by applying the equation described by Elashoff et al. (1982). The other 4 units (SI, AC, TC and DC) consist of double-jacketed glass reactor vessels continuously stirred at 150 rpm by means of a magnetic stirrer (RET control/t IKAMAG). The vessels contain different ports for transit of intestinal content, sampling points, continuous flushing of nitrogen and pH and temperature control. The pH in the colonic units was controlled by addition of 0.5 M NaOH and 0.5 M HCl to keep values of 5.6 ± 0.2 in the AC, 6.3 ± 0.2 in the TC and 6.8 ± 0.2 in the DC. The temperature was kept at 37 ºC by pumping water into the space between the double glass jackets of the reactor units. The computer software controls the addition of the pancreatic juice to the SI unit and the transit times for intestinal content transfer to the AC, TC and DC units. Overall, the model is programmable to sequentially proceed (continuously or feeding the system from 1 to 6 times daily) from the operation of food intake into the stomach throughout the delivery of distal colon content to waste.

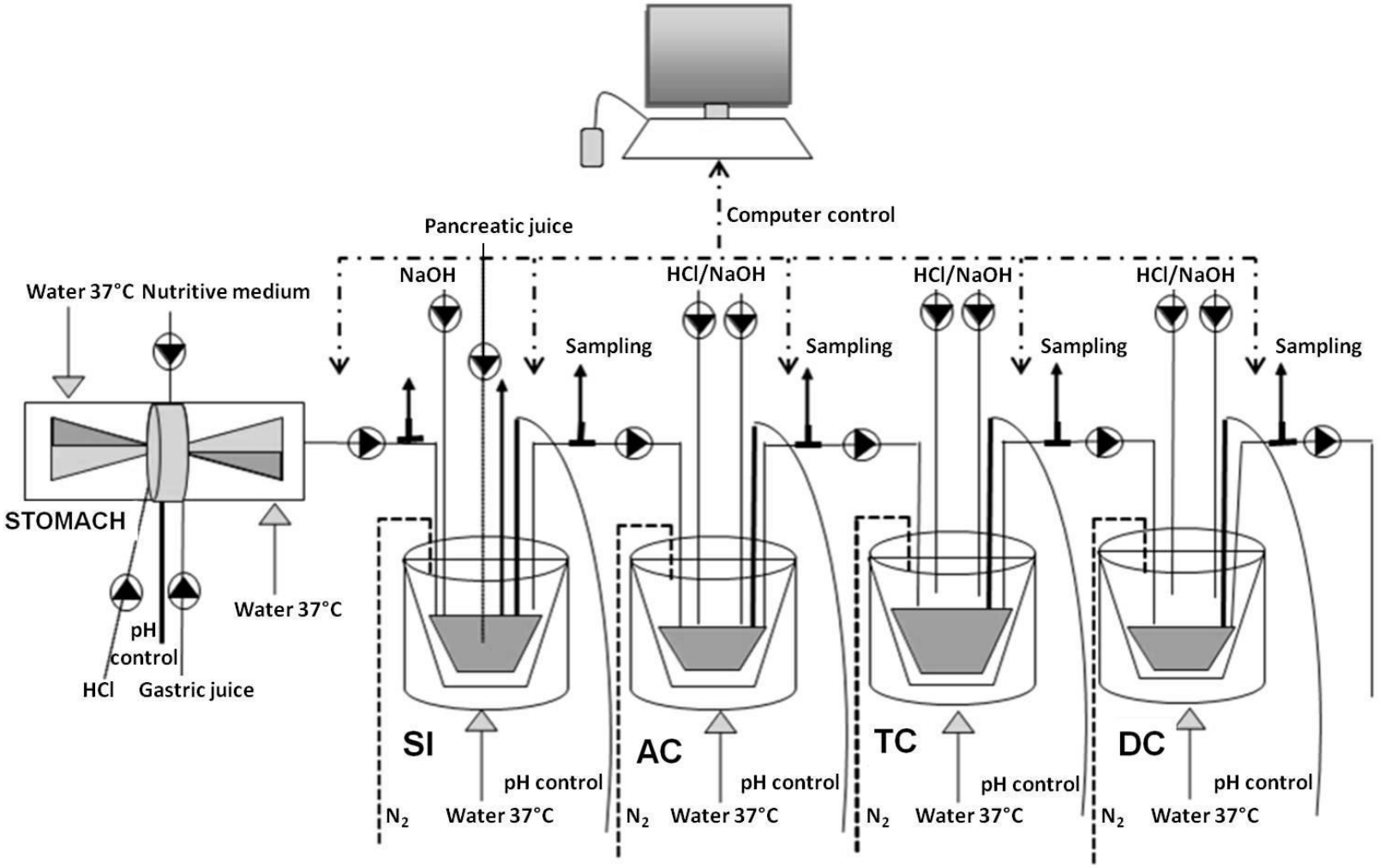

Figure VIII.1. Schematic diagram of the SIMulator GastroIntestinal SIMGI, including the stomach, small intestine (SI) and the ascending (AC), transverse (TC) and descending colon (DC) compartments

VIII.3.2 Microbial community development in the colonic units of the SIMGI

Apart from operating with the five compartments simulating the whole gastrointestinal process, the SIMGI allows both the joint work of the stomach and the small intestine to study food digestion and the direct feeding of the small intestine and the transit to the colonic vessels for studying microbial community development and metabolism. The operating mode to work with units SI, AC, TC and DC was used for the purpose of this study. Therefore, the three colon reactors were filled and pre-conditioned with nutritive medium in a volume of 250, 400 and 300 mL, respectively. The medium contained arabinogalactan (1 g/L), pectin from apple (2 g/L), xylan (1 g/L), potato starch (3 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L). The nutritive medium and the volumes of the colonic reactors to give an overall residence time of 76 h were adapted from the conditions standardized for the SHIME model (De Boever et al. 2000; Molly et al. 1993; Van den Abbeele et al. 2010). The AC, TC and DC units were inoculated with 20 mL of a fresh 20% (w/v) fecal slurry from a healthy volunteer, who had no received any antibiotic treatment in the previous 3 months of the experiment, prepared in anaerobic conditions with sodium phosphate buffer (0.1 M, pH 7.0), containing 1 g/L sodium thioglycolate as reducing agent, as described by De Boever et al. (2000). The inoculated colon units were allowed to equilibrate overnight in batch conditions at 37 °C and at the pH value defined for each compartment. The development and stabilization of the microbial community until steady-state conditions in the three colon units was approached by feeding the small intestine with nutritive medium (75 mL, pH 2) mixed with pancreatic juice (40 mL of a solution of 12 g/L NaHCO3, 6 g/L oxgall dehydrate fresh bile and 0.9 g/L porcine pancreatine) three times a day during 14 days (Van den Abbeele et al. 2010). The small intestine

digestion was performed during 2 h at 37 °C and the content of the vessel was automatically transferred to the following colon compartment (AC) at a flow rate of 5 mL/min, which simultaneously activated the transit of colonic content between the AC, TC and DC compartments at the same flow rate. All the vessels were maintained under anaerobic conditions by continuously flushing N2. During the experimental set up, samples were collected at regular time points (1, 3, 8, 13 and 14 days) from the three colon vessels and stored at −20 °C until further analysis. Microbiological plate counts analyses were performed at the time of sampling.

VIII.3.3 Microbial analyses

VIII.3.3.1 DNA extraction

Microbial DNA extraction of the samples taken from the AC, TC and DC compartments was performed as described by Moles et al. (2013). Briefly, genomic DNA was extracted from samples (1 mL) centrifuged (10000 ×g, 10 min, 4 °C) and the pellet resuspended in an extraction buffer (200 mM Tris–HCl pH 7.5, 0.5% SDS, 25 mM EDTA, 250 mM NaCl, 20 mg/mL lysozyme, 5 mg/mL lysostaphin and 3 M Na acetate), followed by mechanical lysis with glass beads and extraction with phenol/chloroform/isoamyl-alcohol (Sigma-Aldrich). The DNA was precipitated by adding 0.6 volumes of isopropanol, washed with 70% ethanol, allowed to air dry, and finally resuspended in DNase, RNase free water (Sigma-Aldrich). The DNA yield was measured using a NanoDropH ND-1000 UV spectrophotometer (Nano-Drop Technologies).

VIII.3.3.2 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was performed on triplicate samples of 10-fold diluted genomic DNA and analyzed using SYBR green methodology (Bio-Rad Laboratories) with the IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Data analyses were performed with

iQ5 Optical System Software Version 1.1. Primers, amplicon size, annealing temperature for targeted microbial groups (total bacteria, *Enterobacteriaceae*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, the specific phylogenetic groups *Blautia coccoides-Eubacterium rectale* Cluster XIVa, *Ruminococcus* Cluster IV and *Clostridium leptum* subgroup specific cluster IV) have been described previously (Barroso et al. 2013). DNA from *Escherichia coli* DH5α, *L. plantarum* IFPL935, *Bifidobacterium breve* 29M2 and *Bacteroides fragilis* DSM2151 was used for quantification of total bacteria, *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, respectively. For the rest of groups analyzed, samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega), as described previously (Barroso et al. 2013).

VIII.3.3.3 PCR-denaturing gradient gel electrophoresis (DGGE)

Changes in the microbial community were determined using PCR-DGGE essentially as described earlier (Muyzer et al. 1993). Briefly, the PCR fragments were obtained using the primers 968-F (5' AACGCGAAGAACCTTAC-3') and Uni-1401-R (5' CGGTGTGTACAAGACCC-3') (Nubel et al. 1996) to amplify regions of 16 rDNA gene of all bacteria. For DGGE analysis of PCR products, a 40-bp GC clamp was attached to the 3' end of primer 968-F. Then, amplicons were separated by means of a 30–60% denaturant gradient in a polyacrylamide gel using a DCode System (Bio-Rad). The DGGE profiles were digitally normalized by comparison with a home-made standard using InfoQuest FP software (Bio-Rad). Clustering was performed with Pearson correlation and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

VIII.3.3.4 Plate Counts

Appropriate dilutions of samples from each colon region compartment were poured in plates containing a range of selective agar media or broths supplemented with bacteriological agar (1.5%). Thus, dilutions were inoculated into Wilkins-Chalgren agar (Oxoid) for total anaerobes; PCA (Oxoid) for total aerobes; MRS fermentation medium (Pronadisa), which contains neither glucose nor meat extract, supplemented with maltose (1%) for *Lactobacillus*; MacConkey agar (Oxoid) for *Enterobacteriaceae*; Bryant-Burkey medium (Merck) for *Clostridium*; and MRS fermentation medium supplemented with raffinose (1%) and lithium chloride (0.05%) for *Bifidobacterium*. Plates were incubated at 37 °C for 48 h. For anaerobes, *Clostridia* and bifidobacteria, plates were placed in an anaerobic cabinet (BACTRON Anaerobic/Environmental Chamber, SHELLAB).

VIII.3.4 Microbial metabolism analyses

VIII.3.4.1 Short Chain Fatty-Acids (SCFA) determination

Samples from the AC, TC and DC compartments were centrifuged at 13000 ×g for 5 min, the supernatant was filtered and 0.2 µL were injected on a HPLC system (Jasco) equipped with a UV-975 detector and automatic injector. SCFA were separated using a Rezex ROA Organic Acids column (300 × 7.8 mm) (Phenomenex) thermostated at 50 °C following the method described by Sanz et al. (2005). Briefly, the mobile phase was a linear gradient of 0.005 mM sulfuric acid in HPLC grade water, and the flow rate was 0.6 mL/min. The elution profile was monitored at 210 nm, and peaks were identified by comparing retention times with standards. Data acquisition and processing was carried out using a ChromNAV Data System software (Jasco). Calibration curves of acetic, propionic, butyric, formic and lactic acid were built up in the range concentration of 1 to 100 mM.

VIII.3.4.2 Ammonia determination

Ammonia was determined from the supernatant fraction of samples (13000 ×g, 15 min, 4 oC) using an enzymatic kit for ammonia determination (R-Biopharm) based on the method of Gutmann et al. (1974). In this assay, ammonia reacts with 2-oxoglutarate in the presence of glutamate dehydrogenase and NADH. The decrease in NADH, which is proportional to the amount of ammonia, was determined by means of its absorbance at 340 nm.

VIII.4 RESULTS AND DISCUSSION

The computer-controlled multicompartmental dynamic model of the gastrointestinal tract SIMGI described in this study was tested for its capability to reproduce *in vitro* the microbial conditions that characterize the different regions of the human large intestine that have been validated with three-stage culture reactors (Macfarlane et al. 1998; Molly et al. 1994; Van den Abbeele et al. 2010, 2013). The operating conditions of the SIMGI colon compartments in this study have been adapted to the protocols developed for the SHIME (Molly et al. 1993). This model has repeatedly demonstrated its suitability to evaluate long-term effect of food ingredients on modulating the human intestinal microbiota after an initial two-week stabilization period that allows microbial evolution in the reactors from a fecal inoculum to a colon region-specific microbiota (Possemiers et al. 2004; Van den Abbeele et al. 2010). After this stabilization period, the system provides a steady-state environment where the composition and metabolism of the microbial community can be evaluated during long-term experimental dietary interventions (Possemiers et al. 2010; Terpend et al. 2013; Van de Wiele et al. 2007).

Evolution of the microbial community during the two-week stabilization period in the AC, TC and DC compartments of the SIMGI

was analyzed by PCR-DGGE (Fig. VIII.2). Results showed that samples from the three colon compartments clustered together after inoculation and overnight equilibrating conditions (day 1). Nevertheless, variation of the population fingerprints could be observed over time between colon vessels. From day 8 onwards, samples from the same colon compartments clustered together (Pearson correlation coefficients of 95%, 94%, and 91% in the AC, TC and DC compartments, respectively). At the end of the stabilization period, rates of change dropped below 10% for each colon compartment, indicating steady-state microbial compositions (Fig. VIII.2). Differentiation of microbial communities within each colon compartment before reaching the steady state has been described in three-stage microbial reactors (Feria-Gervasio et al. 2014; Van den Abbeele et al. 2010).

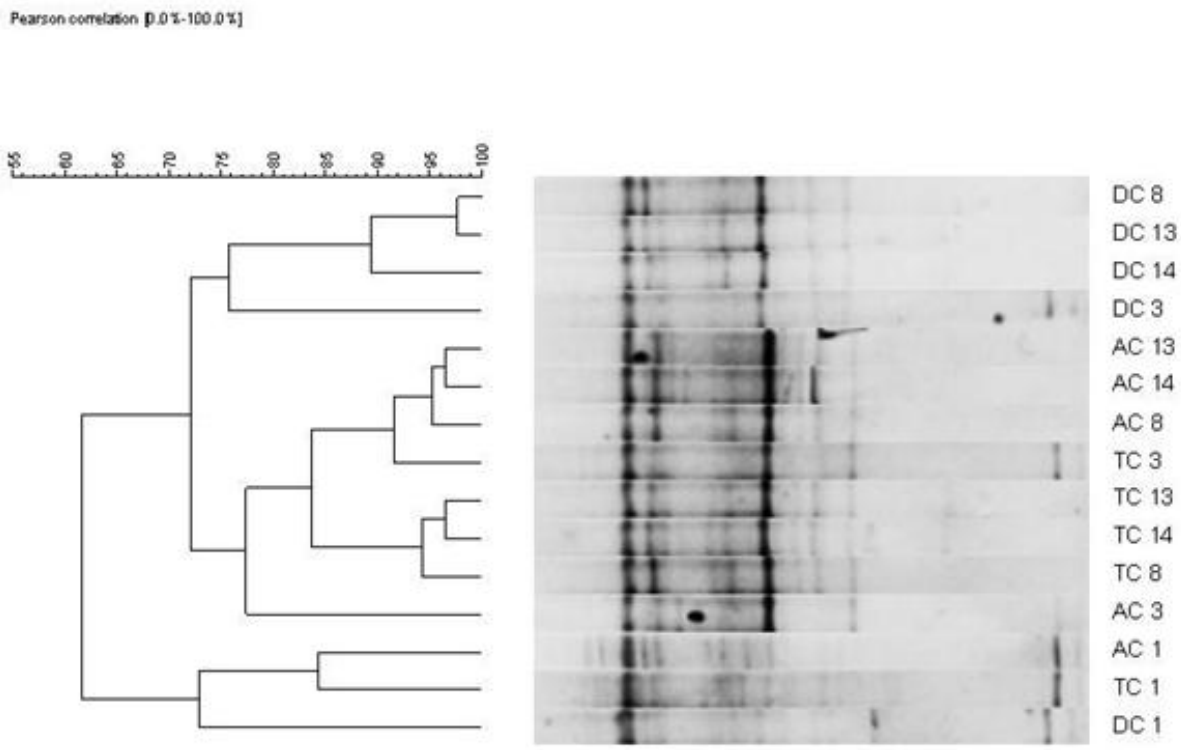

Figure VIII.2. Clustering tree of total bacteria DGGE profiles of samples from the ascending (AC), transverse (TC) and descending (DC) colon compartments at days 1, 3, 8, 13 and 14 after the inoculation of the SIMGI

The composition of the microbial community and the bacterial counts reached during stabilization in the AC, TC and DC compartments were evaluated by qPCR (Table VIII.1). Counts of total bacteria were about 9 log copy number/mL at the onset of the assay in the three colonic regions, remaining steady over the 14 days of the stabilization period. Among the bacterial groups that varied between the inoculum equilibrated overnight in batch conditions (day 1) and the stabilized bacterial community in dynamic conditions (days 13-14), it was observed an overall decrease in counts of *Bifidobacterium* and *Prevotella* and an increase of *Enterobacteriaceae* (Table VIII.1). The microbial community represented by the butyrate-producing groups (cluster IV) *C. leptum* and Ruminoccocus also decreased during stabilization, being the sharpest reduction measured for the *C. leptum* group (2.5 log copy number/mL at 14 days). Differences were also observed within each colon compartment, since these butyrate-producing groups were less represented in the proximal colon vessel (AC) than in the distal vessels (TC and DC) at the end of the stabilization period. Nevertheless, the counts of other butyrate-producing bacteria such as *B. coccoides-E. rectale* group (cluster XIVa) remained equivalent in all colonic compartments (8.1-8.6 log copy number/mL at the end of the stabilization period). On the other hand, *Bacteroides* counts increased in the AC vessel during the time course, being more representative of the AC and TC vessels than the DC vessel (Table VIII.1). Similar results showing an increase in Proteobacteria and *Bacteroides* and a decrease in *Bifidobacterium* and *Clostridium* clusters IV and XIVa, when compared with the fecal inoculum, were determined with the phylogenetic microarray HITChip during the stabilization period of human microbiota in the SHIME (Van den Abbeele et al. 2010) and the TIM-2 (Rajilić-Stojanović et al. 2010) models. Besides, a differentiation in the occurrence of these bacterial groups between the compartments is generally observed in three-stage culture models as a result of the

different conditions established for the three colon vessels. The acidic pH and carbohydrate-rich conditions of the proximal vessel (AC) favoured the predominance of *Bacteroides*, which are characterized by a marked ability to utilize a wide variety of polysaccharides (Ravcheev et al. 2013). Regarding butyrate producer groups, a high abundance of the cluster XVIa group *B. coccoides-E. rectale* in the three colon compartments and a lower occurrence of the cluster IV groups *C. leptum* and Ruminoccocus in the AC compartment than in the TC and DC vessels has been also described at the end of the stabilization period in the SHIME (Barroso et al. 2014; Van den Abbeele et al. 2010).

The microbial community stabilized in the AC, TC and DC compartments of the SIMGI was also evaluated by plate counts. Total bacteria quantitatively reached the steady-state level at mean values of 8 log cfu/mL, which were mainly represented by *Clostridium* and *Enterobacteriaceae* (data not shown). In general, higher counts were obtained with qPCR compared with plate counts, in accordance with the large number of gut bacterial groups that are non-culturable by conventional culture techniques due to their generally fastidious growth requirements (Allen-Vercoe 2013).

Table VIII.1. Mean ± SD of quantitative PCR counts (log copy number/ml) for the different microbial groups analyzed in the ascending (AC), transverse (TC) and descending colon (DC) of the SIMGI during the stabilization period

		Time (days)				
Bacterial group	Compartment	1	3	8	13	14
Universal bacteria	AC	8.79 ± 0.19	9.34 ± 0.45	9.19 ± 0.08	9.26 ± 0.06	9.29 ± 0.04
	TC	9.03 ± 0.53	9.19 ± 0.24	9.47 ± 0.39	8.95 ± 0.03	9.13 ± 0.04
	DC	9.30 ± 0.01	9.36 ± 0.39	9.21 ± 0.36	8.84 ± 0.02	9.12 ± 0.32
Lactobacillus	AC	6.88 ± 1.83	7.50 ± 0.45	7.86 ± 0.06	7.38 ± 0.26	7.44 ± 0.12
	TC	7.57 ± 0.03	7.33 ± 0.08	7.98 ± 0.11	7.58 ± 0.02	7.65 ± 0.03
	DC	7.65 ± 1.94	7.52 ± 2.40	7.36 ± 0.15	7.64 ± 0.05	7.66 ± 0.02
Bifidobacterium	AC	9.67 ± 0.04	8.60 ± 0.03	7.90 ± 0.02	7.43 ± 0.07	7.54 ± 0.06
	TC	9.79 ± 0.31	9.33 ± 0.01	8.28 ± 0.36	7.34 ± 0.14	7.68 ± 0.03
	DC	9.84 ± 0.03	9.40 ± 0.07	8.02 ± 0.30	7.55 ± 0.05	7.58 ± 0.01
Bacteroides	AC	7.11 ± 0.14	7.07 ± 0.06	7.91 ± 0.03	8.18 ± 0.01	8.19 ± 0.16
	TC	7.82 ± 0.13	7.24 ± 0.85	8.02 ± 0.07	7.88 ± 0.11	8.00 ± 0.05
	DC	8.06 ± 0.74	7.71 ± 0.02	7.90 ± 0.04	7.37 ± 0.04	7.39 ± 0.01
Prevotella	AC	6.43 ± 0.02	5.14 ± 0.02	4.67 ± 0.06	4.07 ± 0.01	4.16 ± 0.06
	TC	6.13 ± 0.29	4.91 ± 0.06	4.30 ± 0.01	4.33 ± 0.12	4.45 ± 0.09
	DC	6.59 ± 0.01	4.73 ± 0.02	4.41 ± 0.05	4.34 ± 0.02	4.36 ± 0.08
Enterobacteriacea	AC	4.28 ± 0.18	8.77 ± 0.05	8.74 ± 0.16	8.70 ± 0.01	8.64 ± 0.06
	TC	6.23 ± 1.02	8.62 ± 0.17	8.74 ± 0.10	8.18 ± 0.01	8.32 ± 0.00
	DC	6.11 ± 0.06	7.90 ± 0.16	8.78 ± 0.10	8.04 ± 0.02	8.02 ± 0.02
Blautia coccoides-Eubacterium rectale	AC	8.82 ± 0.05	8.82 ± 0.27	8.53 ± 0.05	8.47 ± 0.03	8.61 ± 0.02
	TC	8.65 ± 0.09	9.04 ± 0.03	8.86 ± 1.17	8.17 ± 0.06	8.47 ± 0.01
	DC	8.91 ± 0.08	8.85 ± 0.35	8.91 ± 0.90	8.03 ± 0.01	8.08 ± 0.06
Clostridium leptum	AC	8.19 ± 0.08	7.00 ± 0.05	5.07 ± 0.02	5.66 ± 0.03	5.70 ± 0.10
	TC	8.03 ± 0.03	7.66 ± 0.05	6.75 ± 0.04	6.46 ± 1.91	6.81 ± 0.01
	DC	7.94 ± 0.09	7.71 ± 0.04	6.81 ± 0.08	6.70 ± 0.05	6.75 ± 0.01
Ruminococcus	AC	6.57 ± 0.00	5.84 ± 0.03	5.04 ± 0.14	5.19 ± 0.03	5.06 ± 0.02
	TC	6.59 ± 0.02	6.40 ± 0.02	5.53 ± 0.18	5.63 ± 0.01	5.73 ± 0.02
	DC	6.87 ± 0.01	6.64 ± 0.00	5.82 ± 0.16	5.83 ± 0.00	5.83 ± 0.02

The metabolism of the microbiota stabilized in the different colonic reactors of the SIMGI model was evaluated by measuring the content of acetic, propionic, butyric, formic and lactic acids (fermentative metabolism) and of ammonium (proteolytic metabolism). Overall, the total SCFAS average molar production up to functional stability was 55.19, 68.14 and 78.68 mM in the AC, TC and DC compartments, respectively. Considering the accumulation of SCFA in the distal compartments observed for three-stage culture reactors without absorption steps (Cinquin et al. 2006; Possemiers et al. 2004), the net SCFA production was highest in the AC compartment in correspondence with the carbohydrate-excess conditions and the higher counts of fermentative bacterial groups, such as *Bacteroides*, in this compartment (Table VIII.1). *Bacteroides* are saccharolytic bacteria characterized by producing acetic, propionic and succinic acids (Flint et al. 2008). The production of acetic, propionic and butyric acids during the microbial stabilization period in the AC, TC and DC compartments is shown in Fig. VIII.3. The evolution pattern of these SCFAs was more similar between the distal compartments (TC and DC) compared to the AC compartment. Thus, acetic acid content decreased in all the vessels up to the stabilization period (14 days) although the initial decrease was sharper in the AC vessel. The decrease of acetic acid production corresponded with the decline of *Bifidobacterium* counts observed in the three compartments, being the fastest decrease also observed in the AC compartment (Table VIII.1). Bifidobacteria are characterized to increase the production of acetic acid when are grown on less readily fermentable substrates (Falony et al. 2006). The decrease in the content of acetic acid can also be explained in the basis of the cross-feeding interactions between colon bacteria (De Vuyst and Leroy 2011). In this sense, the *B. coccoides–E. rectale* group, which prevailed in the SIMGI colonic compartments during the whole stabilization period (Table VIII.1), contains most of the butyrate

producers that use acetic acid as a co-substrate for the enzyme butyryl-CoA : acetate CoA-transferase (Louis and Flint 2009).

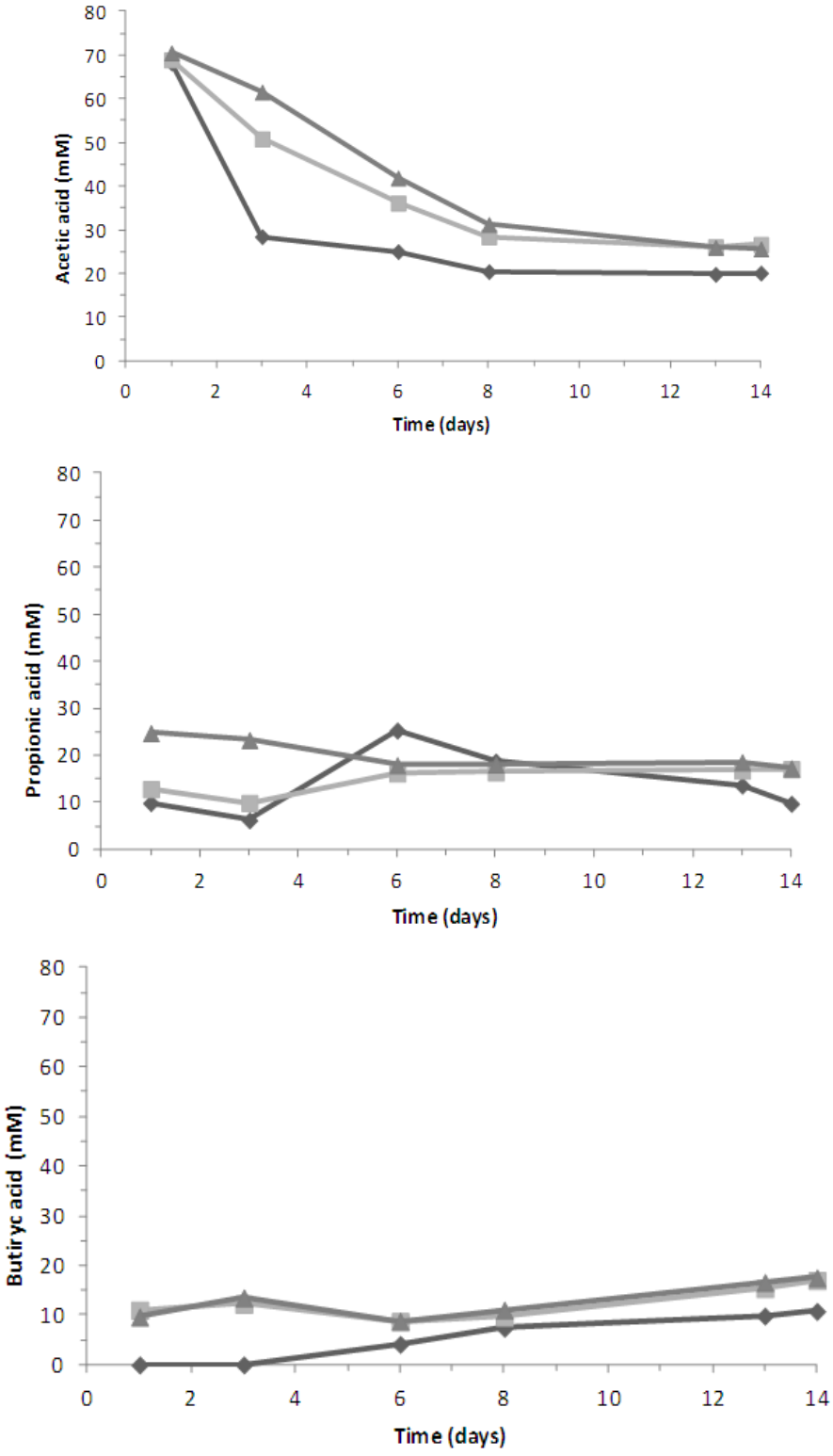

Figure VIII.3. Changes in concentration (mM) of acetic acid, propionic acid and butyric acid in the ascending (AC; diamonds), transverse (TC; squares) and descending colon (DC; triangles) of the SIMGI at different times after inoculation

Regarding other SCFAs, formic acid was detected in the AC compartment from day 8 onwards reaching values of 5 mM at the steady-state (results not shown). Lactic acid production was only detected at day 3 of incubation both in proximal (AC; 6 mM) and distal (DC; 1 mM) colon compartments. In this regard, in fecal samples from healthy donors, lactic acid either is not detected or is present at low concentrations (< 3 mM) due to further metabolism within the colon (Duncan et al. 2007). Initial pH has been point out to play a key role pH upon lactic acid formation and utilization by fecal microbial communities (Belenguer et al. 2007). Besides, lactic acid can be further turned into butyric and propionic acids through cross-feeding by gut bacteria such as *Eubacterium* (Belenguer et al. 2006) and Megasphaera (Shetty et al. 2013), respectively.

Ammonium concentration, a marker for proteolytic activity, was detected to be steady from day 8 of the stabilization period (results not shown). Average values increased along the colonic compartments with levels of 2.8 mM, 4.2 mM and 3.4 mM in the AC, TC and DC vessels, respectively, showing that proteolysis occurred throughout the entire colon compartments. The main pathway of ammonia formation in the human colon is deamination, and bacteria involved in amino acid deamination include species within *Clostridium*, *Bacteroides*, Enterobacterium, and *Lactobacillus* (Scott et al. 2013).

VIII.5 CONCLUSIONS

The fully automation of the SIMGI model allows precise control of the environmental parameters that simulate the gastrointestinal intestinal tract. In this study, we have demonstrated the suitability of this multi-stage dynamic model to reproduce complex and stable microbial communities, which can be differentiated in compartments simulating the three human colon regions. The microbial and functional stabilization observed in the SIMGI model indicates that the system can be used as a tool for studying

the effects of diet or food components on modulating the GIT microbiota and its metabolic activity. The flexible-modulating characteristics of the system and the computer-control of physiological parameters open possibilities for variation of conditions that would allow the simulation of microbial dysbiosis associated to pathological conditions in the SIMGI model. Further advances of the system are addressing the incorporation of devices simulating the gut microbiota-host interactions.

Acknowledgments

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VIII.6 REFERENCES

Allen-Vercoe, E. (2013). Bringing the gut microbiota into focus through microbial culture: recent progress and future perspective. Current Opinion in Microbiology, 16, 625–629.

Aura, A.M., Mattila, I., Hyötyläinen, T., Gopalacharyulu, P., Cheynier, V., Souquet J.M., et al. (2012). Characterization of microbial metabolism of Syrah grape products in an *in vitro* colon model using targeted and non-targeted analytical approaches. European Journal of Nutrition, 52, 833–846.

Barroso, E., Sanchez-Patán, F., Martín-Alvárez, P.J., Bartolomé, B., Moreno-Arribas, M.V., Peláez, C., et al. (2013). *Lactobacillus plantarum* IFPL935 favors the initial metabolism of red wine polyphenols when added to a colonic microbiota. Journal of Agricultural and Food Chemistry, 61, 10163–10172.

Barroso, E., Van de Wiele, T., Jiménez-Girón, A., Muñoz-González, I., Martín-Alvárez, P.J., Moreno-Arribas, M.V., et al. (2014). *Lactobacillus plantarum* IFPL935 impacts colonic metabolism in a simulator of the human gut microbiota during feeding with red wine polyphenols. Applied Microbiology and Biotechnology, 98, 6805–6815.

Belenguer, A., Duncan, S.H., Calder, G., Holtrop, G., Louis, P., Lobley, G.E., et al. (2006). Two routes of metabolic cross-feeding between bifidobacteria and butyrate-producing anaerobes from the human gut. Applied and Environmental Microbiology, 72, 3593–3599.

Belenguer, A., Duncan, S.H., Holtrop, G., Anderson, E., Lobley, G., & Flint, H.J. (2007). Impact of pH on lactate formation and utilization by human fecal microbial communities. Applied and Environmental Microbiology, 73, 6526–6533.

Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim. J., et al. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proceedings of the National Academy of Sciences of the United States of America, 107, 18933–18938.

Cinquin, C., Le Blay, G., Fliss, I., & Lacroix, C. (2006). Comparative effects of exopolysaccharides from lactic acid bacteria and fructooligosaccharides on infant gut microbiota tested in an *in vitro* colonic model with immobilized cells. FEMS Microbiology Ecology, 57, 226–238.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature, 505, 559–563.

De Boever, P., Deplancke, B., & Verstraete, W. (2000). Fermentation by gut microbiota cultured in a simulator of the human intestinal microbial ecosystem is improved by supplementing a soygerm powder. Journal of Nutrition, 130, 2599–2606.

De Vuyst, L., & Leroy, F. (2011). Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production. International Journal of Food Microbiology, 149, 73–80.

Duncan, S.H., Belenguer, A., Holtrop, G., Johnstone, A.M., Lobley, G.E., & Flint, H.J. (2007). Reduced dietary intake of carbohydrate, by obese subjects, results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. Applied and Environmental Microbiology, 73, 1073–1078.

Elashoff, J.D., Reedy, T.J., & Meyer, J.H. (1982). Analysis of gastric-emptying data. Gastroenterology, 83, 1306–1312.

Falony, G., Viachou, A., Verbrugghe, K., & De Vuyst, L. (2006). Cross-feeding between *Bifidobacterium longum* BB536 and acetate converting, butyrate-producing colon bacteria during growth on oligofructose. Applied and Environmental Microbiology, 72, 7835–7841.

Feria-Gervasio, D., Tottey, W., Gaci, N., Alric, M., Cardot, J.M., Peyret, P., et al. (2014). Three-stage continuous culture system with a self-generated anaerobia to study the regionalized metabolism of the human gut microbiota. Journal of Microbiological Methods, 96, 111–118.

Flint, H.J., Bayer, E.A., Rincon, M.T., Lamed, R., & White, B.A. (2008). Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nature Reviews Microbiology, 6, 121–131.

Fritz, J.V., Desai, M.S., Shah, P., Schneider, J.G., & Wilmes, P. (2013). From meta-omics to causality: experimental models for human microbiome research. Microbiome, 1, 14.

Gibson, G.R., Cummings, J.H., & Macfarlane, G.T. (1988). Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Applied and Environmental Microbiology, 54, 2750–2755.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M., (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. Trends in Biotechnology, 30, 591–600.

Gutmann, I., & Bergmeyer, H.U. (1974). Urea. In H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, 2nd Edition (pp. 1791–1801). Academic Press: London.

Hatanaka, M., Nakamura, Y., Maathuis, A.J., Venema, K., Murota, I., & Yamamoto, N. (2012). Influence of *Bacillus subtilis* C-3102 on microbiota in a dynamic *in vitro* model of the gastrointestinal tract simulating human conditions. Beneficial Microbes, 3, 229–236.

Hooper, L.V., Littman, D.R., & Macpherson, A.J. (2012). Interactions between the microbiota and the immune system. Science, 336, 1268e1273.

Kong, F., & Singh, R.P. (2010). A human gastric simulator (HGS) to study food digestion in human stomach. Journal of Food Science, 75, E627-E635.

Louis, P., & Flint, H.J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiology Letters, 294, 1–8.

Macfarlane, G.T., Macfarlane, S., & Gibson, G.R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. Microbial Ecology, 35, 180–187.

Minekus, M., Marteau, P., Havenaar, R., & Huis in't Veld, J.H.J. (1995). A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. Alternatives to Lab Animals, 23, 197–209.

Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonnin, S., Havenaar, R., Marteau, P., et al. (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Applied Microbiology and Biotechnology, 53, 108–114.

Moles, L., Gómez, M., Heilig, H., Bustos, G., Fuentes, S., de Vos, W., et al. (2013). Bacterial diversity in meconium of preterm neonates and

evolution of their fecal microbiota during the first month of life. PLoS One, 8, e66986.

Molly, K., Van de Woestyne, M., & Verstraete, W. (1993). Development of a 5-step multichamber reactor as a simulation of the human intestinal microbial ecosystem. Applied Microbiology and Biotechnology, 39, 254–258.

Molly, K., Van de Woestyne, M., De Smet, I., & Verstraete, W. (1994). Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. Microbial Ecology in Health and Disease, 7, 191–200.

Muyzer, G., De Waal, E.C., & Uitterlinden, G.A. (1993). Profiling of complex populations by denaturating gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59, 695–700.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., et al. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. Journal of Bacteriology, 178, 5636–5643.

Possemiers, S., Verthe, K., Uyttendaele, S., & Verstraete, W. (2004). PCR-DGGE- based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiology Ecology, 49, 495–507.

Possemiers, S., Marzorati, M., Verstraete, W., & Van de Wiele, T. (2010). Bacteria and chocolate: a successful combination for probiotic delivery. International Journal of Food Microbiology, 141, 97–103.

Possemiers, S., Bolca, S., Verstraete, W., & Heyerick, A. (2011). The intestinal microbiome: a separate organ inside the body with the

metabolic potential to influence the bioactivity of botanicals. Fitoterapia, 82, 53–66.

Rajilić-Stojanović, M., Maathuis, A., Heilig, H.G., Venema, K., De Vos, W.M., & Smidt, H. (2010). Evaluating the microbial diversity of an *in vitro* model of the human large intestine by phylogenetic microarray analysis. Microbiology, 156, 3270–3281.

Ravcheev, D.A., Godzik, A., Osterman, A.L., & Rodionov, D.A. (2013). Polysaccharides utilization in human gut bacterium *Bacteroides thetaiotaomicron*: comparative genomics reconstruction of metabolic and regulatory networks. BMC Genomics, 14, 873.

Sanz, M.L., Polemis, N., Morales, V., Corzo, N., Drakoularakou, A., Gibson, G.R., et al. (2005). *In vitro* investigation into the potential prebiotic activity of honey oligosaccharides. Journal of Agricultural and Food Chemistry, 53, 2914–2921.

Scott, K.P., Gratz, S.W., Sheridan, P.O., Flint, H.J., & Duncan, S.H. (2013). The influence of diet on the gut microbiota. Pharmacological Research, 69, 52–60.

Shetty, S.A., Marathe, N.P., Lanjekar, V., Ranade, D., & Shouche, Y.S. (2013). Comparative genome analysis of Megasphaera sp. reveals niche specialization and its potential role in the human gut. PLoS One, 8, e79353.

Terpend, K., Possemiers, S., Daguet, D., & Marzorati, M. (2013). Arabinogalactan and fructo-oligosaccharides have a different fermentation profile in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME ®). Environmental Microbiology, Reports, 5, 595–603.

Van de Wiele, T., Boon, N., Possemiers, S., Jacobs, H., & Verstraete, W. (2007). Inulin-type fructans of longer degree of

polymerization exert more pronounced *in vitro* prebiotic effects. Journal of Applied Microbiology, 102, 452–460.

Van den Abbeele, P., Grootaert, C., Marzorati, M., Possemiers, S., Verstraete, W., Gérard, P., et al. (2010). Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and *Clostridium* cluster IX. Applied and Environmental Microbiology, 76, 5237–5246.

Van den Abbeele, P., Venema, K., Van de Wiele, T., Verstraete, W., & Possemiers, S. (2013). Different human gut models reveal the distinct fermentation patterns of arabinoxylan versus inulin. Journal of Agricultural and Food Chemistry, 61, 9819–9827.

Venema, K., & Van den Abbeele, P. (2013). Experimental models of the gut microbiome. Best Practice and Research Clinical Gastroenterology, 27, 115–126.

Wickham, M., & Faulks, R. (2012). Dynamic Gastric Model. U.S. Patent 8092222.

Wu, G.D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.Y., Keilbaugh, S.A., et al. (2011). Linking long-term dietary patterns with gut microbial enterotypes. Science, 334, 105e108.

IX. EFFECT OF LACTULOSE-DERIVED OLIGOSACCHARIDES ON COLONIC MICROBIOTA DURING THE SHIFT BETWEEN DIETS WITH DIFFERENT ENERGY CONTENTS

Manuscript in preparation

IX.1 ABSTRACT

Dietary habits involving high energy intake are related to the development of overweight and obesity. Prebiotics are recognized to influence the gut microbiota composition and they could be consumed as part of a weight management diet. In this study, we have used the dynamic simulator of the gastrointestinal tract SIMGI to evaluate changes in the microbiological and metabolic characteristics of an overweight-associated colonic microbiota after reducing the diet energy content and its supplementation with the lactulose derived oligosaccharides OsLu. The differentiation and stability of the microbial communities within each colon compartment was reached after two weeks of feeding the system with a high energy diet based on high content of fructose and readily fermentable starches, in order to mimic high processed food and sweet beverage intake. The effect of reducing the energy content loading and the supplementation with OsLu caused variations in bacterial counts generally below 1 log bacteria/mL (except for *Enterobacteriaceace*), indicating that the changes in the chabohydrate content, including the supply with 10 g/L of OsLu, were not able to cause a substantial effect in the colonic bacterial populations of the SIMGI. The reduction of the dietary carbohydrate content caused an effect on the microbial metabolic activity that was characterized by an absence of net butyrate production and an increase in the ammonium content. This shift from fermentative to proteolytic metabolism was not observed when the low-energy diet was supplemented with OsLu. The results obtained in this study indicate that the substitution of easily digestible carbohydrates by OsLu maintains the fermentative functionality of the colonic microbiota, allowing the net production of butyric acid with potential beneficial effects on health, and avoiding a full transition to proteolytic metabolism profiles.

IX.2 INTRODUCTION

Dietary habits involving high energy intake are related to the development of overweight and obesity. Monosaccharides and disaccharides such as fructose and sugar alcohols (sorbitol, lactitol and other polyols), widely used for the formulation of processed foods or beverages, can reach the large intestine when overfeeding of these sugars occurs (Payne et al., 2012a). Analysis of published dietary records reveals that obese children were found to consume significantly more protein and sugars and lower fiber than normal-weight children (Aeberli, et al., 2007). Related to it, there is an increased interest in understanding the possible effects of high energy diets in the intestinal microbiota. However, the heterogeneous and highly personalized human microbiota shows a smaller dietary influence as the inter-individual variation decreases systematic effects even under identical diets (Wu et al., 2011; David et al., 2014). Salonen et al. (2014) described that studies from 14 obese males consuming fully controlled diets supplemented with resistant starch or non-starch polysaccharides and a weight-loss diet revealed that the diet explained around 10% of the total variance in microbiota composition, which was substantially less than the inter-individual variance. All these studies have noted strong individuality of the responses, the extent of which appears to depend on the initial microbiota composition (Korpela et al., 2014). The fact that the broad phylum level changes (Bacteroidetes *vs.* Firmicuttes) have not been found consistently (Ley, 2010; Ravussin et al., 2012) may indicate that relevant changes associated to diet-induced obesity could involve lower taxonomic levels within these phyla (Cox and Blaser, 2013).

In view of the fact that prebiotics are well-recognized to influence the gut microbiota composition, they could be consumed as part of a weight management diet. Genetically obese mice (Cani et al., 2009), diet-induced obese mice and rats (Cani et al., 2007; Pyra et al., 2012), as well

as overweight and obese adults (Parnell and Reimer, 2009) have all been reported to exhibit reduced fat mass following consumption of prebiotics. Sarbini et al. (2014) described the potential of a novel dextran oligosaccharide for obesity management through *in vitro* experimentation. The degree of branching of the compound identified it as a slower-fermenting nutrient that was considered to be advantageous for obese individuals, as energy would be made available more gradually. Recently, the enzymatic synthesis of oligosaccharides derived from lactulose (OsLu) has been aimed for the production of a group of more slowly fermenting prebiotics (Cardelle-Cobas et al., 2008). In addition, the compounds have demonstrated to be selectively fermented by bifidobacteria and lactobacilli and to increase the concentration of short chain fatty acids (Cardelle-Cobas et al., 2012).

In this study we have used the dynamic simulator of the gastrointestinal tract SIMGI described by Barroso et al. (2015). The model simulates the gastric and small intestine digestion and is equipped with three-stage continuous reactors for reproducing the colon region-specific microbiota and its metabolism. The stabilization period in this study has been adapted to simulate an obese-associated microbiota by using a high energy-content medium. Changes in microbiological and metabolic characteristics were assessed after lowering the energy content and the supplementation with the lactulose derived oligosaccharides OsLu used as a potential prebiotic.

IX.3 MATERIALS AND METHODS

IX.3.1 Dynamic simulator of the gastrointestinal tract SIMGI

The gastrointestinal simulator SIMGI was used in the operating mode to work with the units simulating the small intestine (SI) and the ascending (AC), transverse (TC) and descending colon (DC) regions

(Barroso et al., 2014). Therefore, the three colon reactors were filled and pre-conditioned with the nutritive medium that will feed the system during the stabilization period. In this case, the setup was made to recreate an obese-associated microbiota. For this purpose, a starting high energy (HE) medium was used as described by Payne et al. (2012b), which was characterized by a high content of high-glycemic index carbohydrates (digestible starch) and simple carbohydrates (fructose). The HE medium contained arabinogalactan (1 g/L), pectin from apple (2 g/L), xylan (1 g/L), potato starch (6 g/L), maize starch (4 g/L), fructose (6 g/L), glucose (0.4 g/L), yeast extract (3 g/L), peptone (1 g/L), mucin (4 g/L) and L-cysteine (0.5 g/L), that is 45% more fermentable carbohydrates were added to the standard nutritive medium (Barroso et al., 2014) to create the HE diet. The AC, TC and DC units were inoculated with 20 mL of a fresh 20% (w/v) fecal sample from an overweight volunteer, homogenized in anaerobic conditions with sodium phosphate buffer (0.1 M, pH 7.0), containing 1 g/L sodium thioglycolate as reducing agent, as described by De Boever et al. (2000). The development and stabilization of the microbial community until steady-state conditions in the three colon units was approached by feeding the small intestine with nutritive medium (75 mL, pH 2) mixed with pancreatic juice (40 mL of a solution of 12 g/L NaHCO3, 6 g/L oxgall dehydrate fresh bile and 0.9 g/L porcine pancreatine) three times a day during 14 days (Van den Abbeele et al., 2010). The small intestine digestion was performed during 2 h at 37 °C and the content of the vessel was automatically transferred to the following colon compartment (AC) at a flow rate of 5 mL/min, which simultaneously activated the transit of colonic content between the AC, TC and DC compartments at the same flow rate. All the vessels were maintained under anaerobic conditions by continuously flushing N_2.

After the two-week stabilization period of the colonic microbiota, the SIMGI was subjected to a 1-week experiment consisting in removing the maize starch and fructose content and reducing the potato starch content

to 1.5 g/L (low energy medium; LE) and adding 10 g/L of an oligosaccharide mixture derived from lactulose (OsLu) showing prebiotic properties (Cardelle-Cobas et al., 2012). Finally, a 1-week wash-out period was included at the end of the experiment by feeding the SIMGI daily with the LE medium. During the whole study, samples were collected at regular time points from the three colon vessels and stored at −20 °C until further analysis

IX.3.2 Microbiological analyses

IX.3.2.1 DNA extraction and purification

Microbial DNA extraction of the samples taken from the AC, TC and DC compartments was performed as described by Moles et al. (2013). Briefly, samples (1 mL) were centrifuged (10000 ×g, 10 min, 4 °C) and the pellet (resuspended in 200 mM Tris–HCl pH 7.5, 0.5% SDS, 25 mM EDTA, 250 mM NaCl and 3 M Na acetate) was incubated with 20 mg/mL lysozyme and 5 mg/mL lysostaphin (Sigma-Aldrich). Bacterial lysis was completed by mixing with glass beads. The DNA was extracted with phenol/chloroform/isoamyl-alcohol, precipitated by adding 0.6 volumes of isopropanol and finally resuspended in DNase, RNase free water (Sigma-Aldrich). The DNA yield was measured using a NanoDropH ND-1000 UV spectrophotometer (Nano-Drop Technologies).

IX.3.2.2 Quantitative PCR (qPCR)

The quantitative microbiological analysis of samples was carried out by qPCR experiments that were analyzed using SYBR green methodology in a ViiA7 Real-Time PCR System (Life Technologies, USA). Primers, amplicon size, annealing temperature for total bacteria, *Bacteroides*, *Bifidobacterium*, *Enterobacteriaceae*, *Lactobacillus*, *Prevotella*, the specific phylogenetic groups *Blautia coccoides-Eubacterium rectale* Cluster XIVa, *Ruminococcus* Cluster IV and

Clostridium leptum subgroup specific cluster IV) have been described previously (Barroso et al., 2013). DNA from *Escherichia coli* DH5α, *L. plantarum* IFPL935, *Bifidobacterium breve* 29M2 and *Bacteroides fragilis* DSM2151 was used for quantification of total bacteria, *Lactobacillus*, *Bifidobacterium* and *Bacteroides*, respectively. For the rest of groups analyzed, samples were quantified using standards derived from targeted cloned genes using the pGEM-T cloning vector system kit (Promega), as described previously (Barroso et al., 2013). For the analysis of *Akkermansia*, the samples were quantified using standards derived from one clone obtained from the fecal inoculum, amplified with the primers AM1: CAGCACGTGAAGGTGGGGAC and AM2: CCTTGCGGTTGGCTTCAGAT using the conditions described by Collado et al. (2007) and cloned using the pGEM-T cloning vector system kit (Promega, Madison, WI) as described previously (Barroso et al., 2013). The correctness of the *Akkermansia* insert was confirmed by sequence analysis.

IX.3.2.3 PCR-DGGE

For evaluation of the microbial community evolution, DNA was amplified using the universal bacterial primers 968-F and UNI 1401-R described by Nübel et al. (1996). The primer 968-F was synthesized with a 40-bp GC clamp attached to the 3' end. Total volume for PCR reactions was 12.5 µL, composed by 0.75 µL 50 mM MgCl2; 1.25 µL Taq Buffer 10X; 0.25 µL for each primer (10 µM) and dNTPs (10 mM); 0.125 µL Taq Polimerase (5 U/µL), about 100 ng of DNA from each sample and filled up to 12.5 µL with SIGMA water. Amplification protocol was as follows: 94 ºC for 3 min 45 s, 35 cycles of 30 s at 55 ºC (annealing temperature) and 1 min at 72 ºC; and 10 min at 72 ºC. The PCR products (5 µL) were added in a 0.8% agarose gel and analyzed through DGGE by a DCode system equipment (BIORad Lab., USA) using a 9% polyacrylamide gel and a denaturalizing gradient from 30 to 60% of 7 M urea and 40% formamide.

For electrophoresis assay, it was used TAE buffer 0.5X (20 mM Tris, 10 mM acetic acid and 0.5 mM EDTA), at 70 V and 60 ºC for 16 hours. The DGGE profiles were digitally normalized by comparison with a home-made standard using InfoQuest FP software (Bio-Rad). Clustering was performed with Pearson correlation and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

IX.3.3 Microbial metabolism analyses

IX.3.3.1 Short Chain Fatty-Acids (SCFA) determination

Samples from the AC, TC and DC compartments were centrifuged at 13000 ×g for 5 min, the supernatant was filtered and 0.2 µL were injected on a HPLC system (Jasco) equipped with a UV-975 detector and automatic injector. SCFA were separated using a Rezex ROA Organic Acids column (300 × 7.8 mm) (Phenomenex) thermostated at 50 °C following the method described by Sanz et al. (2005). The mobile phase was a linear gradient of 0.005 mM sulfuric acid in HPLC grade water, and flow rate was 0.6 mL/min. The elution profile was monitored at 210 nm and peak identification was carried out by comparison between retention times and standards. For data acquisition and processing it was used a ChromNAV Data System software (Jasco). Calibration curves of acetic, propionic, butyric, formic and lactic acid were built up in the range concentration of 1 to 100 mM.

IX.3.3.2 Ammonium determination

Ammonium was determined directly from the supernatant fraction of samples (13000 ×g, 15 min, 4 oC) using an ammonium ion selective electrode (NH500/2; WTW) and following the manufacture’s instructions.

IX.4 RESULTS AND DISCUSSION

In addition to non-digestible polysaccharides and resistant starches, that reach the large intestine undigested due to resistance to human amylase activity (Shimaya, et al., 2009), simple sugars and digestible starches, when consumed abundantly in the diet, are capable of exceeding intestinal absorption capacity, resulting in high carbohydrate passage into the large intestine, where they are readily available for gut microbial fermentation (Tappy and Le, 2010). Thus, the design of a high energy diet to simulate in the SIMGI an obesity-associated microbiota was based on a significant increase in the content of fructose and readily fermentable starches to the standard nutritious medium employed to feed the SIMGI colonic reactors (Barroso et al., 2015). This diet design represents the increased prevalence of high consumption of refined carbohydrates and fructose-saturated sweeteners that is correlating with the global incidence of obesity (Payne et al. 2012a).

The composition of the microbial community and the bacterial counts reached during the last 3 days of each fermentation period (HE, LE-OsLu and LE) in the AC, TC and DC compartments were evaluated by qPCR (Table IX.1). The end of the stabilization period with the HE diet was characterized in average by higher counts in the distal colon regions of *Bifidobacterium*, *Bacteroides*, *B. coccoides-E. rectale* group, *C. leptum*, *Ruminococcus*, *Akkermansia* and *Enterobacteriacceae*. The most noticeable differences in bacterial counts observed in the SIMGI when comparing the end of the stabilization period with the HE diet and the standard diet (Barroso et al., 2014) were the counts of *Bacteroides* and, particularly, *Enterobacteriaceae* in the three colon compartments that were higher and lower, respectively, with the HE diet in comparison with a standard diet (Barroso et al., 2014). The increase of Proteobacteria is a common feature observed in colonic models (Van den Abbeele et al., 2010; Rajilić-Stojanović et al., 2010). This increase, however, did not take

place during the stabilization of the SIMGI with the HE diet (Table IX.1). *Akkermansia* was the only bacterial group that had not been assayed previously in the SIMGI. The results indicated a predominance of the species in the TC and DC compartments when compared with the AC. This result was already reported in the SHIME by Van den Abbeele et al. (2010). It is important to remark the fact that mucin added to the nutritive media could be relevant for development of *Akkermansia*, as these bacteria depend on mucin as a carbon and nitrogen source (Collado et al., 2007).

After the 15-days stabilization period of the colonic microbiota in the SIMGI with the HE nutritive medium, a shift in diet was carried out by the suppression in the medium of simple carbohydrates and a sharp reduction of the content of readily fermentable starches (LE diet). The carbohydrate content was replaced with the oligosaccharide mixture derived from lactulose (OsLu) developed by Cardelle-Cobas et al. (2008). The incubation of fecal slurries with a similar compound has previously demonstrated prebiotic potential (Cardelle-Cobas et al., 2012). The feeding of the SIMGI with OsLu, however, showed no bifidogenic effect when compared with the HE and LE diets. On the other hand, it was observed a higher content of *Ruminococcus* and lower counts of *Enterobacteriaceae* when comparing with the feeding with the LE diet (Table IX.1). Overall, during the feeding of the SIMGI with the LE-OsLu diet the highest bacterial counts were recorded for the TC compartment, including *Bifidobacterium*, *Bacteroides*, *B. coccoides-E. rectale* group, *C. leptum* and *Ruminococcus*. *Lactobacillus* was also highly represented in the AC compartment at the end of the LE-OsLu diet. Most of the microbial changes observed between the HE and LE-OsLu diets persisted during the feeding with the LE diet, except for the increase of *Bacteroides*, *Prevotella* and *Enterobacteriaceace* observed at the end of the experimental study with the LE diet (Table IX.1). However, except for *Enterobacteriaceace*, differences in bacterial counts bewteen diets

involved variations generally below 1 log units, indicating that the differences in the amount of nutrients, including the supply with 10 g/L of OsLu, were not able to cause a substantial effect in the bacterial counts such as a relevant shifting between sacharolytic and proteolytic populations.

Table IX.1. Mean ± SD of quantitative PCR counts (log copy number/ml) for the different microbial groups analyzed in the ascending (AC), transverse (TC) and descending colon (DC) of the SIMGI at the end of the stabilization period with the high energy (HE) diet and at the end of the feeding with the low energy (LE) diet with and without oligosaccharides derived from lactulose (OsLu).

Bacterial group	Compartment	Diet		
		HE	LE OsLu	LE
Total bacteria	AC	8.83 ± 0.29	8.92 ± 0.42	9.11 ± 0.23
	TC	9.14 ± 0.23	9.19 ± 0.66	9.45 ± 0.17
	DC	9.42 ± 0.18	8.87 ± 0.12	9.26 ± 0.10
Lactobacillus	AC	7.36 ± 0.32	7.74 ± 0.38	7.74 ± 0.46
	TC	7.41 ± 0.08	7.45 ± 0.18	7.49 ± 0.23
	DC	7.20 ± 0.13	6.83 ± 0.11	6.94 ± 0.33
Bifidobacterium	AC	6.45 ± 1.04	5.22 ± 0.04	7.10 ± 0.15
	TC	7.30 ± 0.52	7.62 ± 0.48	7.87 ± 0.25
	DC	7.45 ± 0.16	7.46 ± 0.25	7.92 ± 0.14
Bacteroides	AC	8.66 ± 0.38	9.00 ± 0.33	9.52 ± 0.16
	TC	9.29 ± 0.13	9.51 ± 0.08	9.51 ± 0.10
	DC	9.32 ± 0.22	8.98 ± 0.27	9.24 ± 0.12
Blautia coccoides-	AC	7.38 ± 1.00	7.19 ± 0.39	8.05 ± 0.41
Eubacterium rectale	TC	8.50 ± 0.14	8.46 ± 0.11	8.56 ± 0.07
	DC	8.69 ± 0.18	7.97 ± 0.42	8.16 ± 0.18
Clostridium leptum	AC	2.70 ± 0.77	2.11 ± 0.28	2.28 ± 0.10
	TC	7.24 ± 0.16	6.92 ± 0.10	6.96 ± 0.09
	DC	7.00 ± 0.20	6.48 ± 0.24	6.69 ± 0.03
Ruminococcus	AC	1.73 ± 1.23	3.43 ± 1.78	1.94 ± 0.94
	TC	4.67 ± 0.08	4.85 ± 0.06	5.53 ± 0.36
	DC	5.51 ± 0.01	5.47 ± 0.20	5.89 ± 0.13
Prevotella	AC	4.16 ± 0.61	4.54 ± 1.20	5.18 ± 0.05
	TC	4.62 ± 0.46	4.96 ± 0.62	5.56 ± 0.28
	DC	4.72 ± 0.21	4.46 ± 0.09	4.56 ± 0.01
Akkermansia	AC	2.20 ± 0.79	2.80 ± 0.16	3.18 ± 0.26
	TC	3.57 ± 0.05	3.52 ± 0.55	3.39 ± 0.41
	DC	3.46 ± 0.04	3.89 ± 0.57	3.91 ± 0.13
Enterobacteriaceae	AC	2.84 ± 0.17	5.56 ± 0.28	7.43 ± 0.13
	TC	6.18 ± 0.03	7.56 ± 0.08	7.75 ± 0.16
	DC	5.95 ± 0.19	6.72 ± 0.23	7.88 ± 0.16

The qPCR counts were in agreement with the qualitative assessment of biodiversity analyzed by PCR-DGGE. The analysis was carried out to compare the differentiation of microbial communities within each colon compartment and to qualitatively detect changes to the biodiversity as a function of varied substrate availability. Fig. IX.1 shows the microbial community profiles of the AC, TC and DC compartments at the last day of each intervention period of the SIMGI with the HE diet followed by the LE diet supplemented with OsLu and finally with only the LE diet. The results showed that samples from the three colon compartments clustered together independently of the carbohydrate content of the diets. This feature is consequence of the characteristics of three-stage fermentation models that allow reproducing differences from proximal, characterized by acidic pH and carbohydrate-excess conditions, to distal colonic regions showing a carbohydrate-depleted and non-acidic environment (Macfarlane and Macfarlane, 2007).

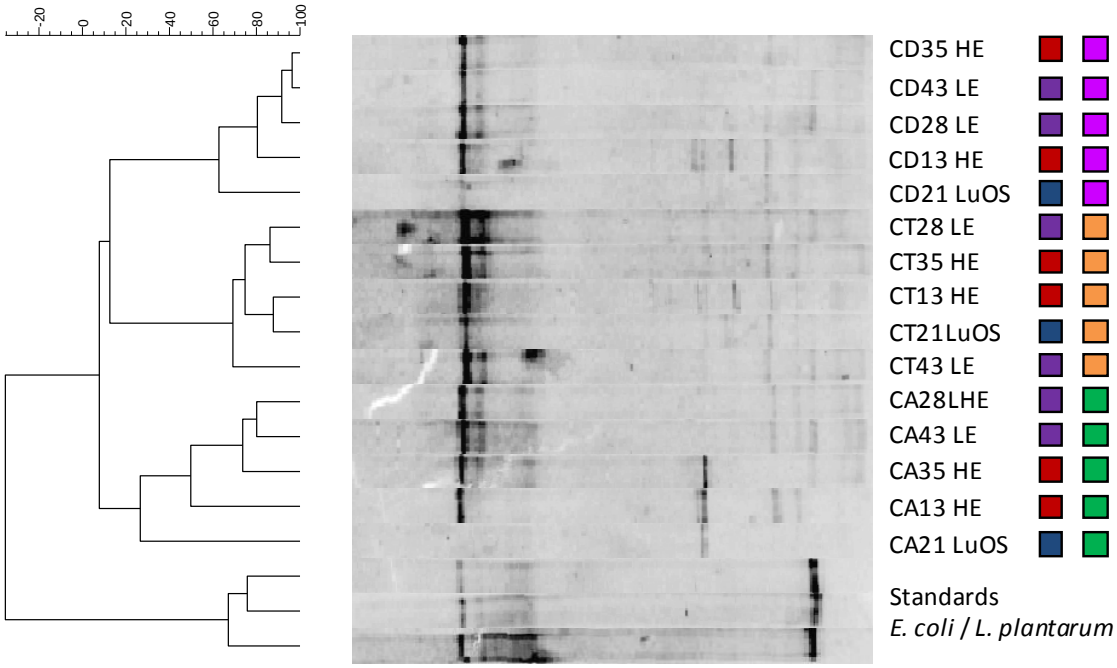

Figure IX.1. Clustering tree of total bacteria DGGE profiles of samples from the ascending (CA; green), transverse (CT; orange) and descending (CD; fucsia) colon compartments at the last days of feeding the SIMGI with the HE (red), LE (violet) and OsLu (blue) diets.

The results of metabolic activity, analyzed as the production of SCFA, lactic acid and ammonium, of the microbial community during the last 3 days of each fermentation period (HE, LE-OsLu and LE) in the AC, TC and DC compartments are shown in Table IX.2. Except for lactic and formic acids, the SCFA and ammonium concentrations gradually increased from the AC to the DC compartment because of the accumulation of products in the system, consistent with operation of three-stage culture reactors without absorption steps (Cinquin et al., 2006; Possemiers et al., 2004). Lactic and formic acids were only produced in the AC compartment and the latest only in the HE and LE-OsLu diets. Lactic acid can be further metabolised within the colon and turned into butyric and propionic acids through cross-feeding by gut bacteria (Duncan et al., 2004; Reichardt et al., 2014). Likewise, formic acid is a component in the mutualistic interaction between fermentative bacteria and syntrophic metabolizers and has an important role in anaerobic metabolism via interspecies cross-feeding interactions (Louis et al., 2014). *Bifidobacterium* has been described to produce formic acid from several carbohydrate sources, whereas some *Lactobacillus* species undergo a metabolic shift towards acetate and formate production, at the expense of lactate production, when growing on non-digestible oligosaccharides (Tabasco et al., 2014). The metabolic shift has been associated with more ATP production, resulting in a more efficient use of the available energy source (Van der Meulen et al., 2004).

The effect of reducing the energy nutrient loading on microbial metabolic activity was characterized by an overall 2-fold decrease in the average content of total SCFA, mainly associated to acetic acid changes, of the three colon compartments with the LE diet compared to the HE intake period. Within the SCFA analyzed, propionate production was the least affected by nutrient load, whereas butyrate production was practically stopped with the LE diet (Table IX.2). These results point toward the microbial utilization of the butyrate via methanogenesis or

sulfate reduction (Worm et al., 2014). The absence of net butyrate production could be restored by supplementating the LE diet with OsLu. Additionally, the shift from high to low energy medium caused a 2-fold increase in the ammonium content of the distal colon compartments (TC and DC) and a remarkable 5.5-fold increase in the proximal colon compartment (AC). This shift from fermentative to proteolytic metabolism was not observed when the LE diet was supplemented with OsLu (Table IX.2; Fig. S1). The SFCA and ammonium results could be compared with *in vivo* data from obese subjects, where a significant decrease of SCFA, particularly acetate and butyrate, and an increase of proteolytic products were observed when the individuals consumed diets high in protein and reduced in total carbohydrates (Russell et al., 2011; Levy and Borenstein, 2014). There is evidence from both humans and animal models that dietary supplementation with non-digestible carbohydrates can decrease protein fermentation in the large intestine, which concurs with a decrease in the genotoxicity of faecal water (Windey et al., 2012).

Table IX.2. Changes in concentration (mM) of SCFA and ammonium in the ascending (AC), transverse (TC) and descending colon (DC) of the SIMGI at the end of the stabilization period with the high energy (HE) diet and at the end of the feeding with the low energy (LE) diet with and without oligosaccharides derived from lactulose (OsLu).

Compound		HE	LE OsLu	LE
Total SCFA	AC	88.24 ± 2.53	59.17 ± 12.36	36.54 ± 0.74
	TC	110.04± 14.50	94.30± 16.12	50.89± 1.36
	DC	120.35 ± 2.50	86.88 ± 0.21	54.80 ± 0.21
Acetic acid	AC	45.46 ± 3.79	33.91 ± 2.08	22.81 ± 0.86
	TC	57.63 ± 6.27	46.43 ± 7.55	30.84 ± 0.33
	DC	62.84 ± 1.61	44.03 ± 0.44	30.70 ± 0.47
Propionic acid	AC	16.36 ± 0.30	17.27 ± 4.84	11.06 ± 0.14
	TC	28.05 ± 4.64	25.60 ± 4.94	16.69 ± 1.16
	DC	32.17 ± 1.32	26.48 ± 0.55	20.55 ± 0.34
Butyric acid	AC	21.39 ± 0.43	13.58 ± 4.29	0.05 ± 0.08
	TC	27.53 ± 4.24	22.28 ± 3.63	3.36 ± 0.13
	DC	25.34 ± 0.43	16.38 ± 0.77	3.34 ± 0.62
Lactic acid	AC	3.85 ± 0.00	2.53 ± 0.00	2.62 ± 0.05
	TC	nd	nd	nd
	DC	nd	nd	nd
Formic acid	AC	3.12 ± 1.32	2.90 ± 0.00	nd
	TC	nd	nd	nd
	DC	nd	nd	nd
Ammonium	AC	4.05± 1.19	7.07± 3.09	22.39± 4.97
	TC	34.80± 15.79	50.61± 6.44	50.44± 6.99
	DC	55.68± 19.23	67.50± 5.15	63.10± 2.47

Nd: not detected

In conclusion, the results obtained in this study indicate that except for *Enterobacteriaceae*, characterized by becoming great competitors in carbohydrate scarcity, stability of the microbial populations was the dominant pattern. Community structure clusters were predominately a function of the specific-region colonic conditions, suggesting that community structures are relatively robust with little substantial change during shifts in nutrient supply. Furthermore, metagenomic studies are consistently showing that inter-individual differences in gut microbiota in terms of microbial composition can be over 90% (Dorrestein et al., 2014), but that there is an assembly of functional communities that share similarities in their metabolic pathways (Shafquat et al., 2014). It implies that distinct microbial species may be responsible for specific functions and adapt themselves to environment and diet affecting human homeostasis and health status. The results obtained in this study indicate that substitution of easily digestible carbohydrates by OsLu allows the development of a fermentative functionality, maintaining the net production of butyric acid with potential beneficial effects on health, and avoiding a full transition to proteolytic metabolism profiles.

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IX.5 REFERENCES

Aeberli I, Zimmermann MB, Molinari L, Lehmann R, l'Allemand D, Spinas GA, Berneis K (2007b). Fructose intake is a predictor of LDL particle size in overweight schoolchildren. Am J Clin Nutr 86: 1174-1178.

Barroso E, Cueva C, Peláez C, Martínez-Cuesta MC, Requena T (2014). Development of human colonic microbiota in the computer-controlled dynamic SIMulator of the GastroIntestinal tract SIMGI. WT Food Sci. Technol. 61: 283-289.

Barroso E, Sanchez-Patán F, Martín-Alvárez PJ, Bartolomé B, Moreno-Arribas M.V, Peláez C, Requena T, van de Wiele T, Martínez-Cuesta MC (2013). *Lactobacillus plantarum* IFPL935 favors the initial metabolism of red wine polyphenols when added to a colonic microbiota. J. Agric. Food. Chem. 61:10163–10172.

Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM (2007). Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia 50(11):2374-83.

Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, Geurts L, Naslain D, Neyrinck A, Lambert DM, Muccioli GG, Delzenne NM (2009). Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58:1091-103.

Cardelle-Cobas A, Martínez Villaluenga C, Villamiel M, Olano A, Corzo N (2008). Synthesis of oligosaccharides derived from lactulose and Pectinex Ultra SP-L. J. Agric. Food Chem 56: 3328−3333.

Cinquin C, Le Blay G, Fliss I, Lacroix C (2006). Comparative effects of exopolysaccharides from lactic acid bacteria and

fructooligosaccharides on infant gut microbiota tested in an *in vitro* colonic model with immobilized cells. FEMS Microbiol. Ecol. 57:226–238.

Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S (2007). Intestinal integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl Environ Microbiol 73(23):7767-70.

Cox LM, Blaser MJ. Pathways in microbe-induced obesity (2013). Cell Metab 17(6):883-94.

David L.A, Maurice C.F, Carmody R.N, Gootenberg D.B, Button J.E, Wolfe B.E, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature 505:559–563.

De Boever P, Deplancke B, Verstraete W (2000). Fermentation by gut microbiota cultured in a simulator of the human intestinal microbial ecosystem is improved by supplementing a soygerm powder. J. Nutr. 130:2599–2606.

Dorrestein PC, Mazmanian SK, Knight R (2014). Finding the missing links among metabolites, microbes, and the host. Immunity 40:824-32.

Duncan SH, Louis P, Flint HJ (2004). Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 70:5810–5817

Korpela K, Flint H, Johnstone AM, Lappi J, Poutanen K, Dewulf E, Delzenne N, de Vos W, Salone A. (2014). Gut microbiota signatures predict host and microbiota responses to dietary interventions in obese individuals. PLoS One 9: e90702.

Levy R, Borenstein E (2014).Metagenomic systems biology and metabolic modeling of the human microbiome: from species composition to community assembly rules. Gut Microbes 5:265-70.

Ley RE (2010). Obesity and the human microbiome. Curr. Opin. Gastroenterol. 26:5–11.

Louis P, Hold GL, Flint HJ (2014). The gut microbiota, bacterial metabolites and colorectal cancer. Nat Rev Microbiol 12(10):661-72.

Macfarlane GT, Macfarlane S (2007). Models for intestinal fermentation: association between food components, delivery systems, bioavailability and functional interactions in the gut. Curr Opin Biotechnol 18(2):156-62.

Moles L, Gómez M, Heilig H, Bustos G, Fuentes S, de Vos W., Fernández L, Rodríguez JM, Jiméne E. (2013). Bacterial diversity in meconium of preterm neonates and evolution of their fecal microbiota during the first month of life. PLoS One 8, e66986.

Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W, Backhaus H (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178:5636–5643.

Parnell JA, Reimer RA (2009). Weight loss during oligofructose supplementation is associated with decreased ghrelin and increased peptide YY in overweight and obese adults. Am J Clin Nutr 89:1751-9.

Payne AN, Chassard C, Lacroix C (2012a). Gut microbial adaptation to dietary consumption of fructose, artificial sweeteners and sugar alcohols: implications for host-microbe interactions contributing to obesity. Obes Rev 13:799–809.

Payne AN, Chassard C, Banz Y, Lacroix C (2012b). The composition and metabolic activity of child gut microbiota demonstrate differential adaptation to varied nutrient loads in an *in vitro* model of colonic fermentation. FEMS Microbiol Ecol 80:608-23.

Possemiers S, Bolca S, Verstraete W, Heyerick A (2011). The intestinal microbiome: a separate organ inside the body with the metabolic potential to influence the bioactivity of botanicals. Fitoterapia 82:53–66.

Pyra KA, Saha DC, Reimer RA (2012) Prebiotic fiber increases hepatic acetyl CoA carboxylase phosphorylation and suppresses glucose-dependent insulinotropic polypeptide secretion more effectively when used with metformin in obese rats. J Nutr 142(2):213-20.

Rajilić-Stojanović M, Maathuis A, Heilig HG, Venema K, De Vos WM, Smidt H (2010). Evaluating the microbial diversity of an *in vitro* model of the human large intestine by phylogenetic microarray analysis. Microbiology 156:3270–3281.

Ravussin Y, Koren O, Spor A, LeDuc C, Gutman R, Stombaugh J, Knight R, Ley RE, Leibel RL (2012). Responses of gut microbiota to diet composition and weight loss in lean and obese mice. Obesity (Silver Spring) 20:738–747.

Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, Flint HJ, Louis P (2014). Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. ISME J 8(6):1323-1335.

Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, Duncan G, Johnstone AM, Lobley GE, Wallace RJ, Duthie GG, Flint HJ (2011). High-protein, reduced-carbohydrate weight-loss diets promote

metabolite profiles likely to be detrimental to colonic health. Am. J. Clin. Nutr. 93:1062-1072.

Salonen A, Lahti L, Salojärvi J, Holtrop G, Korpela K, Duncan SH, Date P, Farquharson F, Johnstone AM, Lobley GE, Louis P, Flint HJ, de Vos WM (2014). Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men. ISME J 8(11):2218-30

Sanz ML, Polemis N, Morales V, Corzo N, Drakoularakou A, Gibson GR, Rastall RA (2005). *In vitro* investigation into the potential prebiotic activity of honey oligosaccharides. J. Agric. Food. Chem. 53:2914–2921.

Shafquat A, Joice R, Simmons SL, Huttenhower C (2014). Functional and phylogenetic assembly of microbial communities in the human microbiome. Trends Microbiol 22(5):261-6.

Shimaya S, Shimoyama T, Fukuda S, Matsuzaka M, Takahashi I, Umeda T, Chinda D, Saito D, Sakamoto J, Nagura T, Danjo K, Nakaji S (2009). The recovery rate at the human terminal ileum of an orally administered non-digestive oligosaccharide (raffinose). Int J Food Sci Nutr 60: 344-351.

Tabasco R, Fontecha J, Fernández de Palencia P, Peláez C, Requena T (2014). Competition mechanisms of probiotic bacteria: fermentative metabolism and colonization. LWT - Food Sci. Technol 55:680-684.

Tappy L, Le KA (2010). Metabolic effects of fructose and the worldwide increase in obesity. Physiol Rev 90: 23-46.

Van den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gérard P, Rabot S, Bruneau A, El Aidy S, Derrien M, Zoetendal E, Kleerebezem M, Smidt H, Van de Wiele T (2010). Microbial

community development in a dy-namic gut model is reproducible, colon region specific, and selective for *Bacteroidetes* and *Clostridium* cluster IX. Appl. Environ. Microbiol. 76: 5237-5246.

Van der Meulen R, Makras L, Verbrugghe K, Adriany T, De Vuyst, L. (2006). *In vitro* kinetic analysis of oligofructose consumption by *Bacteroides* and *Bifidobacterium* spp. indicates different degradation mechanisms. Appl. Environ. Microbiol. 72(2):1006-1012.

Windey K, de Preter V, Verbeke K (2012). Relevance of protein fermentation to gut health. Mol. Nutr. Food Res 56:184–196.

Worm P, Koehorst JJ, Visser M, Sedano-Núñez VT, Schaap PJ, Plugge CM, Sousa DZ, Stams AJ (2014). A genomic view on syntrophic versus non-syntrophic lifestyle in anaerobic fatty acid degrading communities. Biochim Biophys Acta 1837(12):2004-16.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis DJ (2011). Linking long-term dietary patterns with gut microbial enterotypes. Science 334:105-108.

IX.6 SUPPLEMENTARY MATERIAL

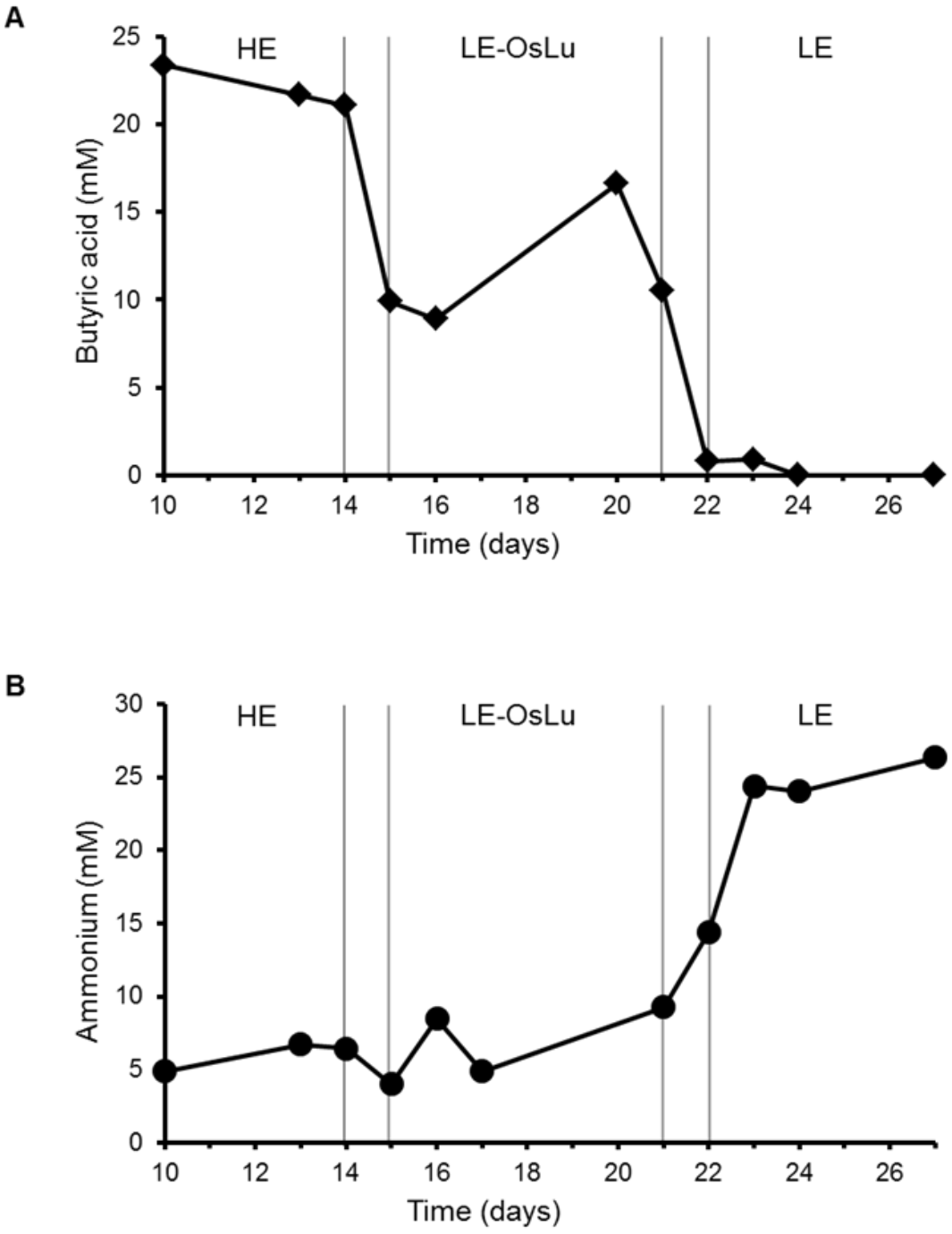

Fig. S1. Changes in concentration (mM) of butyric acid (diamonds) and ammonium (circles) in the ascending colon (AC; circles) of the SIMGI during the periods of stabilization with the HE, OsLu and LE diets.

X. CONCLUSIONS/CONCLUSIONES

CONCLUSIONS

First

Polyphenols of cranberry, grape seed and red wine extracts possess antimicrobial activity against some bacterial groups of the intestinal microbiota, in particular over *Bacteroides*, *Prevotella* and *Blautia coccoides-Eubacterium rectale*. Among the tested extracts, grape seed polyphenols showed the highest antimicrobial effect.

Second

Lactobacillus plantarum IFPL935 has a protective role on some intestinal bacteria affected by the presence of red wine polyphenols, *Bacteroides* and *Bifidobacterium* in batch incubations, and on the butyrate-producing groups *Ruminococcus*, *B. coccoides/E. rectale* and *Clostridium leptum*, particularly during dynamic simulation. Likewise, *L. plantarum* IFPL935 has a positive impact on the butyric acid production in distal colon regions.

Third

Lactobacillus plantarum IFPL935 has demonstrated that in presence of a complex human colonic microbiota is able to initiate the metabolism of flavan-3-ols, giving rise to the intermediate compounds diphenylpropan-2-ol, (5-(3′-hydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3’-hydroxyphenyl)-valeric acid, and favours the formation of end metabolites such as phenylpropionic acids.

Fourth

The moderate intake of red wine during four weeks does not significantly modify the composition of the human oral and intestinal microbiota. Overall, the large inter-individual variability in the microbial

profiles has a stronger influence for the grouping of samples than the possible influence of wine intake.

Fifth

The dynamic *in vitro* gastrointestinal simulator SIMGI is capable to stabilize and reproduce the composition and fermentative activity of the ascending, transverse and descending colon regions of the human microbiota.

Sixth

The feeding during one week with lactulose-derived oligosaccharides (OsLu), as a prebiotic ingredient in a low carbohydrate-content diet, does not produce changes in the composition of the colonic microbiota developed in the SIMGI. The addition of OsLu allows to maintain the microbial fermentative functionality, represented by net butyric acid production in the ascending colon region, and to decrease proteolysis.

CONCLUSIONES

Primera

Los polifenoles de los extractos de arándano rojo, pepita de uva y vino tinto poseen actividad antimicrobiana frente a algunos grupos bacterianos de la microbiota intestinal, especialmente sobre *Bacteroides*, *Prevotella* y *Blautia coccoides-Eubacterium rectale*. Entre los extractos evaluados, los polifenoles de pepita de uva mostraron el mayor efecto antimicrobiano.

Segunda

Lactobacillus plantarum IFPL935 ejerce un papel protector sobre bacterias intestinales que se ven afectadas por la presencia de los polifenoles de vino tinto, como *Bacteroides* y *Bifidobacterium* en incubaciones estáticas, y los grupos productores de ácido butírico *Ruminococcus*, *B. coccoides/E. rectale* y *Clostridium leptum*, particularmente en la simulación dinámica. Asimismo, *L. plantarum* IFPL935 tiene un impacto positivo sobre la formación de ácido butírico en regiones colónicas distales.

Tercera

Lactobacillus plantarum IFPL935 ha demostrado que en presencia de una microbiota colónica compleja inicia el metabolismo de flavan-3-oles, mediante la formación de compuestos intermediarios como dihidroxifenilpropan-2-ol, 5-(3′-hidroxifenil)-γ-valerolactona y ácido 4-hidroxi-5-(3′-hidroxyfenil)valérico, y favorece la formación de metabolitos finales como ácidos fenilpropiónicos.

Cuarta

El consumo moderado de vino tinto durante cuatro semanas no causa diferencias significativas en la composición de la microbiota oral e

intestinal humana. En general, la alta variabilidad interindividual de los perfiles microbianos tiene mayor efecto en el agrupamiento de las muestras que el que pudiera ejercer el consumo de vino.

Quinta

El simulador gastrointestinal dinámico *in vitro* SIMGI reproduce de manera estable la composición y la actividad fermentativa de la microbiota representativa de las regiones del colon humano ascendente, transverso y descendente.

Sexta

La alimentación durante una semana con oligosacáridos derivados de lactulosa (OsLu) como ingrediente prebiótico en una dieta baja en carbohidratos no ocasiona cambios en la composición de la microbiota colónica desarrollada en el SIMGI. La administración de OsLu permite mantener la funcionalidad fermentativa representada por la producción neta de ácido butírico en la región ascendente del colon y reducir la proteólisis.